2019

Early Life Stress and Immune Responses in Adult Rat Kidneys

Ijeoma E. Obi
University of Alabama at Birmingham

EARLY LIFE STRESS AND IMMUNE RESPONSES IN ADULT RAT KIDNEYS

by

IJEOMA EBELECHUKWU OBI

JENNIFER S. POLLOCK, CHAIR
DAVID M. POLLOCK
MICHELLE V. FANUCCHI
JAMES F. GEORGE
LISA M. CURTIS

A DISSERTATION

Submitted to the graduate faculty of The University of Alabama at Birmingham,
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

BIRMINGHAM, ALABAMA

2019

EARLY LIFE STRESS AND IMMUNE RESPONSES IN ADULT RAT KIDNEYS

IJEOMA EBELECHUKWU OBI

PATHOBIOLOGY AND MOLECULAR MEDICINE

ABSTRACT

Globally, human studies show overwhelming associations between adverse childhood experiences and cardiovascular disease (CVD) and CVD risks throughout adult life. As early as 6 years old, there are significant associations between childhood adversity and inflammation, and those association are observed throughout adult life as well. Over a decade ago, rodent models were used to establish the importance of the immune cells in hypertension, which is the major risk factor in developing CVD. Although these associations in humans are important, they pose several limitations that can be overcome by the use of animal models to study the molecular mechanisms that are mediating CVD. This dissertation characterizes the renal immune state of the rodent model used to study early life adversity, and then begins to explore how the immune cells contribute to blood pressure elevation in response to a hypertensive stimulus.

To achieve the goals in this dissertation, the maternal separation (MatSep) rodent model of early life stress (ELS) was utilized. In this model, ELS was induced by separating male pups from the dam from postnatal day (PD) 2 to PD 14, for 3 hours a day. Non-separated littermates served as controls. Renal immune responses as a result of the induced ELS were studied in adulthood at 12 weeks of age.

The major findings from this study are that MatSep induces programming of the innate immune system by displaying increased renal levels of IL-1β in the distal tubules and increased TLR-4 immunopositive interstitial cells in the renal medulla. Also, MatSep kidneys display increased neutrophil activation, greater numbers of CD44 immunopositive cells, and increased number of proliferating cells in the renal medulla. When given an immune challenge with LPS, MatSep rats displayed a heightened renal cytokine and chemokine gene expression which were not observed in littermate controls. Interestingly, nitric oxide (NO) blockade with LNAME led to significantly lower blood pressure compared to control, and LNAME also protected against LNAME-induced tubular injury.

In summary, characterizing the renal immune profile in this dissertation allows for future in-depth studies of immune mechanisms mediating CVD and CVD risks.

Keywords: Cardiovascular disease, early life stress, maternal separation, rats, kidney, nitric oxide

DEDICATION

I dedicate this dissertation to myself, Ijeoma Ebelechukwu Obi. To my strength, my resilience, my patience, my stamina, my pain, my joy, my discipline, my laughter, for without these virtues there would be no finale.

To my parents Mr. Sampson Emenike Obi and Mrs. Esther Ifeyinwa Obi for their unconditional love, support, prayers, and for the sacrifices that they made particularly during the last year and half of my PhD.

To my sisters Chika Nwadiogo Obi, Ogechukwu Anulika Obi, and Ozioma Ngozika Obi for being my much-needed distraction and balance during my program.

To the Almighty God for being my strong foundation, my friend, my confidant, and my guide.

ACKNOWLEDGMENTS

I would like to thank my advisor, Jennifer Pollock for the opportunity to work in your lab and for all the experiences I gained during my years in the lab.

To my committee members, Drs. James George, Lisa Curtis, Michelle Fanucchi, and David Pollock, you are the best committee any graduate student would wish for. You all supported my journey wholeheartedly and helped me grow tremendously. I am very grateful for that. Dr. George, I will never forget when you told me in front of your lab members that I am now part of your lab family, and you never treated me any less. You gave me your resources and spent countless time helping me with my data. You are indeed a great mentor. Dr. Pollock, thank you for providing me with the resources for my project. It meant a lot to me.

To members of my lab, thank you for making my experience a fun one. To Drs. Carmen de Miguel, Paramita Patti, and Daian Chen I only have great memories. To my fellow graduate students, Randee, Luke, Partick, and Dr. Fox thank you for being there for me and for all the wonderful discussions and for the late nights we spent working together in the lab. To Dr. Xiaofen, I'll miss working in the histology room with you. To Miller and Jack, I thank you for lending a helping hand. To members of David Pollock's lab, Dr. Eman Gohar, I appreciate the collaborative opportunity you offered me. To Dr. Brian Becker, I appreciate all the times you helped me with all of my telemetry work. You patiently troubleshot a lot of the problems I encountered, and you were always

willing to help me when I needed it most. To Dr. Malgorzata Kasztan, you are one of the most hardworking scientists I know. To Crystal, Dingguo, and Reham you all are amazing students and you made my life as a graduate student enjoyable. To Dr. Jermaine Johnston, we started this graduate student life together from Georgia and we finished strong.

A few unexpected people helped me during the toughest part of graduate school. To Dr. Kelly Hyndman, thank you for having an answer to every question and for your great heart. To Dr. Anupam Agarwal, your kind gesture toward me and my family, I will never forget. To Dr. Scott Ballinger you were a great help in my time of great need. I pray that as you showed me great kindness that you will reap exceptionally great kindness as well.

Finally, I will like to acknowledge my family. To my father, Sampson Obi, you are the best father any girl could ever wish for. The sacrifices you've made and continue to make for me are unbelievable. You have pushed me to be and do my best especially when no one is watching. You have covered me spiritually and emotionally. You have given me much confidence and I cannot imagine my life without a father like you. To my mother, Esther Obi, my prayer warrior, I appreciate you for loving me and encouraging me. I love you and dad so very much and I hope I have made you proud and will continue to make you proud.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

STATEMENT OF THE PROBLEM

Early life stress (ELS) is an independent risk factor in developing cardiovascular disease in humans (Alastalo et al., 2012; Alastalo et al., 2013; Cho, Bower, Kiefe, Seeman & Irwin, 2012; Felitti et al., 1998). Epidemiological studies associate ELS with increases in pro-inflammatory mediators as well (Coelho, Viola, Walss-Bass, Brietzke & Grassi-Oliveira, 2014; Danese, Pariante, Caspi, Taylor & Poulton, 2007; Pace et al., 2006). However, it is unknown whether ELS-dependent immune cell activation and pro-inflammatory factors mediate cardiovascular disease risk. The overall goal of this proposal is to determine the involvement of the immune system in cardiovascular disease risk using an animal model of ELS.

Hypertension is one of the major risk factors for developing cardiovascular disease. Immune cells such as dendritic cells, macrophages, and T cells are involved in blood pressure elevation as shown by several animal models (De Miguel, Das, Lund & Mattson, 2010; Franco et al., 2007; Guzik et al., 2007; Itani et al., 2016; Kirabo et al., 2014; Rudemiller et al., 2016; Zhang et al., 2016) and in humans (Imakiire et al., 2007).

Previous studies from our laboratory utilized the rat model of ELS, maternal separation (MatSep), to show differences in blood pressure and T cell numbers in adult rats in response to a hypertensive stimulus. The MatSep model involves separating pups from the dam from postnatal day 2 (PD2) to PD14 for 3 hours a day. These pups are then studied as adults at 12 weeks old. Normally reared littermates are used as controls.

Findings from those studies showed significant blood pressure elevation in MatSep rats and significant proportions of $CD3^+$ T cell infiltration in MatSep kidneys compared to controls. These findings suggest that MatSep displayed a sensitization towards higher blood pressure in response to a hypertensive stimulus, as well as increased $CD3^+$ T cells in the kidneys. Whether the higher blood pressure in MatSep rats is the result of the increased $CD3^+$ T cells in the kidneys is yet to be determined.

It is well established that $CD3^+$ T cell subsets ($CD4^+$ and $CD8^+$ T cells) infiltrate the kidneys during hypertension; however, several studies have begun to delineate the involvement of $CD4^+$ and $CD8^+$ T cells in hypertension. For example, $CD8^+$ T cells, but not $CD4^+$ T cells were necessary for angiotensin II (AngII)-induced hypertension (Trott et al., 2014) and in salt-sensitive hypertension by direct stimulation of sodium transporter in the distal tubule (Liu et al., 2017). We have evidence that in Nω-Nitro-L-Arginine Methyl Ester (LNAME)-induced hypertension, $CD8^+$ T cells may be higher in MatSep kidneys. In this proposal, we designed experiments to test the hypotheses that MatSep induces priming of immune responses in the kidney, and that ELS-induced immune cell contribute to the observed blood pressure elevation in MatSep rats.

AIM 1: To characterize the renal inflammatory state in adult male rats

AIM 1a: To determine the effect of MatSep on circulating and renal immune cell numbers, renal immune cell activation status, and cytokine levels.

AIM1b: To determine the effect of MatSep on chemokine and cytokine gene

expression changes in response to an immune challenge.

AIM 2: To test the hypothesis that in MatSep rats, LNAME activates renal $CD8^+$ T cells leading to exaggerated elevation in blood pressure.

AIM 2a: To determine if MatSep induces an exaggerated blood pressure response in LNAME-induced hypertension compared to control rats.

AIM 2b: To determine if renal $CD8^+$ T cells are activated in MatSep rats compared to control rats in response to LNAME.

AIM 2c: To determine if blocking $CD8^+$ T cells would lower LNAME-induced blood pressure elevation in MatSep rats.

CHAPTER 2

INTRODUCTION

ADVERSE CHILDHOOD EXPERIENCES (ACEs)

Adverse childhood experiences (ACEs) are defined as traumatic events having long-lasting negative impact on adult health (Figure 1). These range from physical, sexual and verbal abuse in childhood to stressful family, social, economic, and environmental changes with lasting mental or emotional distress to the child. The Centers for Disease Control (CDC)-Kaiser Permanente ACE Study is one of the largest investigations of childhood abuse and neglect linked to later-life health and well-being. These groundbreaking population studies conducted at Kaiser Permanente from 1995 to 1997 involved two waves of data collection and a cohort of over 17,000 Health Maintenance Organization members from southern California receiving physical exams and completion of confidential surveys regarding their childhood experiences as well as current health status and behaviors. ACEs were grouped into abuse (emotional, physical, and sexual), neglect (emotional and physical), and household dysfunction (substance abuse, mental illness, domestic violence, criminal household member, and parental marital discord). The results showed that two-thirds of the participants studied had experienced at least one ACE and one in five participants reported three or more ACEs in the first 18 years of life. The ACE score, a total sum of the different categories of ACEs reported by participants, is used to assess cumulative childhood stress. Study findings

repeatedly reveal a graded dose-response relationship between ACEs and negative health and well-being outcomes across the life span (Felitti et al., 1998).

Early life stress (ELS) is the general nomenclature used to describe environmental or behavioral stress during the perinatal and postnatal period that includes humans and animal models, while the term ACE is used exclusively in reference to humans. Rodent models of ELS, such as maternal separation (MatSep) in rats or maternal separation with weaning (MSEW) in mice, are established behavioral stress models used to decipher mechanistic links to increased risk of disease in adult life, particularly depression and anxiety (Francis, Caldji, Champagne, Plotsky & Meaney, 1999). Within the last 10 years, ELS-specific mechanisms are beginning to be elucidated.

This introduction will focus on: (1) epidemiological associations between ACEs and CVD risks, (2) evidence for associations between ACE exposures and immune-mediated and/or vasoactive pathways, (3) rodent models of ELS-induced hypertension risk, (4) pro-inflammatory mediators and vasoactive factors as mechanisms of ELS-induced hypertension risk, and (5) role of the kidneys in hypertension and influences of the immune cells.

EPIDEMIOLOGICAL ASSOCIATIONS BETWEEN ACEs AND CVD RISKS

In recent years, several studies have demonstrated strong associations between ACEs and CVD risks in adult life. ACEs have been identified as a CVD risk factor, although underappreciated. CVD is the leading cause of death resulting in one out of every three deaths worldwide (Benjamin et al., 2017). It is estimated that by the year

2030, the total cost of treating CVD worldwide would be $1,044 billion, creating a global burden(Benjamin et al., 2017). Various factors increase the risk for CVD. These factors

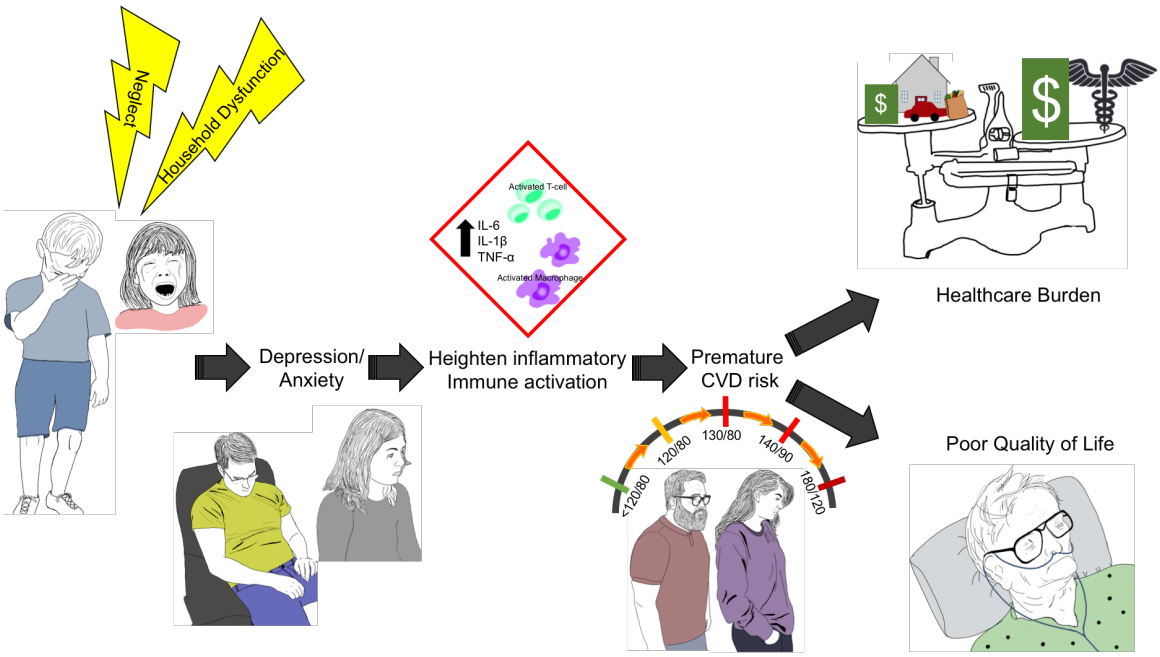


Figure 1. ACEs, such as neglect, abuse, and household dysfunction, in humans are associated with increased risk of hypertension and developing CVD. Majority of studies investigating the association of childhood adversity and CVD suggests detrimental effects of ACEs (depression/anxiety) may induce well known mechanisms that are involved in the development of hypertension such as inflammation and the associated immune mediators that lead to premature CVD risk and, ultimately, decreased quality of life and increased health burden in adulthood.

are hypertension, high cholesterol, diabetes, smoking, obesity, family history, poor dietary life style, and age. Studies in humans show significant associations between ACEs and CVD as well as ACEs and CDV risks. This section would examine these epidemiological associations.

Cardiovascular disease

Dong et al conducted a study in humans to examine the relationship between ACEs and the risk of developing IHD (Dong et al., 2004). Also, the authors assessed the effects of graded associations between ACEs and the risk of IHD. ACEs was defined by childhood abuse (emotional, physical and sexual abuse), neglect (emotional and physical), and household (substance abuse, mental illness, domestic violence, criminal household member, and parental marital discord). IHD was self-reported and defined as a "yes" response to: if one has been told they have had a heart attack, any chest pain or heavy pressure in the chest, or if they used nitroglycerine. This study is unique in the sense that previous studies showed that ACEs are strongly associated with traditional and psychological risk factors for IHD such as smoking, obesity depression and physical inactivity; however, Dong et al examined direct association between ACEs and IHD. The results from the study showed that individuals who experienced one type of ACEs had a significantly greater prevalence of having IHD. This finding was true in all subset of ACEs examined in the study except for parental marital discord. As expected, the prevalence of IHD was significantly higher to a much greater extent in individuals who had either the traditional (diabetes, hypertension, smoking, physical inactivity, and BMI) or psychological (depression and anger) risk factors for IHD. Furthermore, the study revealed that as the number of ACEs reported by an individual increased, the likelihood that an individual reported having IHD increased by 20 percent. However, after adjusting for traditional and psychological risk factors, that likelihood decreased to 10 percent. The authors reanalyzed the data by including individual over the age of 50 in order to predict if the prevalence of IHD increases as the number of ACEs increases. This analysis was

done because after the age of 50, the prevalence of IHD increases dramatically. Indeed, there was a positive relationship between the increased number of ACEs and higher prevalence of IHD in patients over 50. The findings from older patients further validated the direct association between ACEs IHD (Dong et al., 2004).

In the Helsinki cohort study, the effect of war on cardiovascular health was studied in Finnish adults who were temporary separated from their parents to foster parents living in Sweden and Denmark during world war II (WWII) (Alastalo et al., 2012). The temporary separation of children from their biological parents was a form of childhood stress even though they were separated for their safety. The study utilized data from the Finnish National Archives which contained the age and duration of parental separation, and the National Hospital Discharge Register (HDR) and the Causes of Death Register (CDR) to determine coronary events and stroke in participants. Separated adults were at a higher risk of taking medication for coronary heart disease (CHD) compared to non-separated adults. Interestingly, the risk of taking medication for CHD was maintained even after adjusting for adult socioeconomic status and educational achievement. Of the deceased participants, the mortality and morbidity rates were also higher in war evacuees than non-evacuees. Furthermore, the prevalence of CHD was determined for the duration of separation and at different age categories at separation (infancy: less than 2 years of age; toddlerhood: 2-4 years of age; early childhood: 4-7 years of age; and school age: 7-11 years of age). Indeed, adults who were separated during early childhood had a significantly higher CHD morbidity than non-separated individuals. Additionally, a higher prevalence of CHD was observed in individuals that

were separated for 1-2 years and for more than 3 years compared to non-separated individuals.

Another study by Godwin and Stein set out to determine associations between childhood trauma and physical disorders among U.S adults (Goodwin & Stein, 2004). The National Comorbidity survey used in the study was based on non-institutionalized U.S populations. Child abuse was defined sexual abuse (rape and sexual molestation), physical abuse and seriously neglected as a child. Data analyses revealed that sexual abuse was significantly associated with cardiac disease (heart attack or other serious heart trouble) after adjusting for demographic characteristics alone. After further adjusting for multiple factors such as demographic characteristics, anxiety and depressive like behaviors, alcohol and substance dependence, the previously observed association between sexual abuse and cardiac disease remained significant in females but not in males. No significant associations were observed between physical abuse or severe childhood neglect with cardiac disease in all participants and in males and females.

A household survey conducted around England between April and July in 2013, was done in order to determine if ACEs has any effect on developing chronic illness in the study participants (Bellis, Hughes, Leckenby, Hardcastle & Lowey, 2014). In this study, 9 categories of ACEs were assessed: physical, verbal and sexual abuse, parental separation, exposure to domestic violence, and growing up with members of the household that suffered mental illness, alcohol abuse, drug abuse or incarceration. Among the diseases assessed were CVD and stroke. CVD was defined as coronary heart disease and heart attack. In participants with greater than 4 ACEs, there were significant increase in CVD and stroke compared to those with no ACEs, 1 ACEs or 2-3 ACEs.

There was a trend towards significance in participants with 2-3 ACEs and the risk for CVD (Bellis, Hughes, Leckenby, Hardcastle & Lowey, 2014).

In the Study of Women's Health Across the Nation (SWAN) community-based study in midlife women, the authors tested whether women with prior history of childhood physical and sexual abuse had higher carotid intima media thickness (IMT) by ultrasound imaging (Thurston et al., 2014). The women were sampled from the Boston; Chicago; the Detroit area; Los Angeles; Newark, New Jersey; Pittsburg, Pennsylvania; and Oakland, California. Findings from the study showed that childhood sexual abuse alone was associated with greater IMT. Interestingly, no associations were observed in participants who were only sexually abused in their adult life with IMT.

Another female only study by Rich-Edwards et al utilized data from the Nurses' Health Study 2 (NHS2) to test whether childhood abuse physical and sexual abuse are associated with CVD in women only (Rich-Edwards et al., 2012). The cardiovascular endpoint was based on questionnaire in which participants indicated whether they have been diagnosed with myocardial infarction (MI) or angina, and stroke or transient ischemic attack (TIA) and each participant's medical records were reviewed by a physician in a blinded manner. After adjusting for childhood risk factors like race, parental education at time of birth, and parental CVD history, women who experienced physical abuse had increased risk for CVD events compared to women who were not physically abused during childhood. This was the case for women who reported severe physical abuse. Women who reported mild or moderate physical abuse were not associated with increased risk for CVD. It is interesting to note that after adjusting for lifestyle and CVD risk factors especially adult BMI, hypertension, and diabetes, the

observed increased risk for CVD risk in women who experienced severe physical abuse was attenuated. This finding suggests that the association between severe abuse and increased risk for CVD is mainly driven by CVD risk factors. Sexual abused women had increased risk for CVD as well after adjusting for childhood risk factors; however, this finding as only observed in women who reported forced sex abuse.

The World Mental Health Surveys investigated a culturally diverse samples from 10 different countries to determine whether ACEs and early onset of mental disorders were independently associated with the increased risk of chronic diseases (Scott et al., 2011). The countries represented were Americans (Columbia, Mexico, and the United States), Europe (Belgium, France, Germany, Italy, Netherlands, and Spain), and Asia (Japan). ACEs assessed were physical and sexual abuse, neglect, parental death, parental divorce, other parental loss (adoption, foster care, or leaving home before the age of 16), parental mental disorder, parental substance abuse, parental criminal behavior, family violence, and family economic adversity. There were significant associations between physical and sexual abuse, parental death, other parental loss, parental mental disorder, and parental substance abuse and the prevalence of heart disease. Additionally, exposure to more than 2 ACEs led to a graded increase in heart disease.

Overall, these studies provide compelling evidence that: (1) ACEs are significantly associated with an increased risk for CVD events in adult life. (2) there is a dramatic increase in the prevalence of CVD as the number of ACEs increases. (3) there are gender specific differences in ACEs associations with the prevalence of CVD, and (4) in a culturally diverse study, the associations between ACEs and the prevalence of CVD still persists.

Cardiovascular disease risk

Diabetes

In 2017, 7.6% of U.S population were diagnosed with diabetes (American Diabetes Association, 2018). This percentage translated to 24.7 million people in the U.S. It is estimated that the total national cost of treating diabetes is 327 billion dollars, thus treating diabetes alone imposes a huge financial burden (American Diabetes Association, 2018). Several studies have reported strong associations between ACEs and diabetes. In a longitudinal study conducted in the Add Health Wave IV cohort, young adults between ages 24 to 34 were studied for associations between childhood abuse and neglect prior to age 12, and diabetes and prediabetes (Duncan, Auslander, Bucholz, Hudson, Stein & White, 2015). Diabetes was defined as HbA1c ≥6.5%, fasting glucose ≥126mg/dL, non-fasting blood glucose ≥200mg/dl, participants reported taking anti-diabetic medication, and/or participants reported that a health care provider diagnosed them with diabetes or high blood glucose. Prediabetes was defined as HbA1c 5.7%-6.4%, and/or fasting glucose 100-125mg/dL. Their findings showed that 4.1% of men who had diabetes were sexually abused 3 or more times, 1.3% of men who were prediabetic, and 1.2% of men without diabetes. Diabetic women had an increased prevalence of reporting that they were emotionally abused at least 1 to 2 times compared to women who were prediabetic or non-diabetic. Also, women who reported that they were neglected at least 1 to 2 times showed a greater risk for prediabetes.

Another study that utilized state-based data from noninstitutionalized U.S adults who are less than 18 years old in order to determine their health condition and behavioral risk factors, the study found that participants who reported that they experienced one to

three and four to six ACEs had greater odds of diabetes compared to participants who did not experience any ACEs. No associations were found in participants who experienced 7 to 9 ACEs. (Gilbert et al., 2015). Furthermore, the psychological traumatic effect of the Second Lebanon War on type 1 diabetes were studied in Israeli children between ages 0-17 years (Zung et al., 2012). The incidence of type 1 diabetes was assessed by comparing the incidence of type 1 diabetes 4 years before the war (pre-war) and two years after the war (post-war). The incidence of type 1 diabetes was higher in the no-war zones compared to the war zones during the pre-war years; however, in the post-war years the incidence of type 1 diabetes became greater in the war zones compared to the no-war zones. Interestingly, the observed increased incidence of type 1 diabetes post-war was only significant in boys than in girls. Even though it is not fully understood how psychological stress leads to type 1 diabetes, the authors suggests that it may be due to dysregulation of immune cells in response to acute and chronic stress (Zung et al., 2012).

A more recent study from the Whitehall cohort aimed to examine the associations between ACEs and diabetes and whether depression and cardiometabolic dysregulations mediated the observed associations in British civil servants (Deschenes, Graham, Kivimaki & Schmitz, 2018). ACEs was defined as being hospitalized for four or more weeks, parental divorce, unintentional parental unemployment, parental mental illness or alcoholism, physical abuse, observing frequent parental arguments or fights, living in an orphanage home, and maternal separation for more than 1 year or more, all incidences occurring from 0-16 years of age. Depression was determined using the Center for epidemiologic Studies Depression Scale (CES-D), while cardiometabolic characteristics were defined as central obesity (men: waist circumference ≥102cm; women: ≥88cm), low

HDL cholesterol (< 1.03 mm/L in men and < 1.30 mm/L in women), high triglyceride levels (> 1.7mmol/L), poor glycemic control (fasting blood glucose > 5.6 mmol/L), and hypertension (blood pressure >130/85 mmHg). Overall, ACEs were associated with increased risk of developing diabetes, and for every increase in the number of ACEs exposure led to approximately 11 percent increase in the odds of developing diabetes. An important finding in this study was the statistically significant evidence showing that the direct association between ACEs and diabetes is dependent on depressive symptoms and cardiometabolic characteristics and indication of two mechanistic pathways.

The NHSII study revealed a higher risk of diabetes in women who were moderately and severely abused physically, experienced unwanted sexual touching, and forced sex at least once or repeatedly (Rich-Edwards et al., 2010). Finally, women who experienced both physical and sexual abuse had a greater risk of diabetes than women who experienced either type of abuse alone. Additionally, in a review article by Huffhines et al that aimed to determine associations between ACE and trauma as risk factors in developing diabetes, the authors conclusions from 38 articles further supports that ACE increases the risk of adult diabetes (Huffhines, Noser & Patton, 2016).

Obesity

Rich-Edwards et al showed from the NHSII cohort that girls who experienced severe physical abuse and repeated forced sex had a higher BMI compared girls who were not abused by the end of adolescence (Rich-Edwards et al., 2010). Additionally, by adulthood the trajectory between girls who experienced severe physical abuse and repeated forced sex grew wider when compared to girls who were not abused by the end of adolescence (Rich-Edwards et al., 2010).

In the Duncan et al study, participants were grouped into BMI categories: underweight/normal weight (BMI $\leq$ 24.9kg/m^2), overweight (BMI 25.0-29.9 kg/m^2), obese class 1 (BMI 30.0-34.9 kg/m^2), obese class II (BMI 35.0-39.9 kg/m^2), and obese class III (BMI $\geq$ 40.0 kg/m^2) (Duncan, Auslander, Bucholz, Hudson, Stein & White, 2015). The finding revealed a significant association between only physical abuse and all BMI categories compared to participants who were underweight/normal weight in both men and women. No associations were found between sexual abuse, emotional abuse, and neglect in both men and women (Duncan, Auslander, Bucholz, Hudson, Stein & White, 2015).

Metabolic syndrome

Metabolic syndrome is a group of several conditions that exist at the same time, thus increasing the risk of CVD. These conditions include glucose intolerance, high blood pressure, abdominal obesity, and high cholesterol. Data from the Midlife Development in the U.S. study (MIDUS) revealed that individuals who experienced childhood trauma had more symptoms of metabolic syndrome and a greater risk of being diagnosed with metabolic syndrome (Lee, Tsenkova & Carr, 2014). There also existed sex differences in the association between different categories of childhood trauma and having symptoms of metabolic syndrome in adulthood. In men, increase in severity of only physical and emotional abuse showed associations to metabolic syndrome; whereas, in females only sexual abuse showed an increased risk (Lee, Tsenkova & Carr, 2014). In addition, participants in the Dunedin Multidisciplinary Health and Development study also showed that socially isolated children and children who were raised in low socioeconomic environment were at greater risk of having metabolic risk markers (overweight, high

blood pressure, high cholesterol, low high-density cholesterol, high glycated hemoglobin, and low maximum oxygen consumption levels) in adulthood (Danese et al., 2009). The Carolina Abecedarian Project also supports the association between increase metabolic syndrome in individuals exposed to childhood adversity (Campbell et al., 2014).

Age

The process of ageing involves decline in physical and cognitive capabilities. The Avon Longitudinal Study of Parents and Children (ALSPAC) cohort was used to test if childhood parental low socioeconomic status was associated with decline in cognitive and physical capabilities in adult life (Anderson et al., 2017). Physical capability was assessed by a height-adjusted grip strength test, a timed chair rise, a timed one-leg standing balance test with eyes closed, and a 3-minute timed walk test. Cognitive capabilities were defined as verbal fluency, logical memory, delayed logical memory, digit backward, digit symbol coding, and spot-the-word tests. Parental low socioeconomic status was associated with lower physical and cognitive capabilities (Anderson et al., 2017).

Hypertension

According to the 2017 American College of Cardiology (ACC)/American Heart Association (AHA) hypertension guidelines, hypertension affects more than 70 million people between the ages of 45-75 in the United States and approximately 15 million of the affected individuals have uncontrolled hypertension (Khera et al., 2018). Hypertension is one of the most significant risk factors for developing CVD and is also multi-faceted in the mechanistic links to progression of the disease (Merai et al., 2016).

Findings from participants in the female only Nurses' Health Study II (NHS II) cohort revealed an increased risk of incident adult hypertension (blood pressure > 140/70 mmHg) with sexual touching and activity as well as physical abuse compared to women who reported no abuse (Riley, Wright, Jun, Hibert & Rich-Edwards, 2010). Also, the risk of hypertension was stronger with increased level of abuse (Riley, Wright, Jun, Hibert & Rich-Edwards, 2010). In a longitudinal cohort study conducted in healthy participants who experienced childhood adversity before age 18, results from ACE questionnaires were divided into 3 major categories: childhood abuse, childhood neglect, and household dysfunction (Su et al., 2015). The longitudinal trajectories of systolic and diastolic blood pressure with age were steeper in individuals with ACEs than individuals with no ACEs. In addition, this higher blood pressure trajectory occurred in a graded dose response with the increasing number of ACEs in an individual (Su et al., 2015).

Apart from childhood abuse and neglect, the effect of war as an ACE on hypertension also has been studied in older adults. The Helsinki cohort studies consist of people who were born in Helsinki, Finland, during World War II (WWII) between 1934-1944. During WWII, some Finnish children were evacuated temporarily to foster parents in Sweden without being accompanied by their parents. About 60 years later, the effect of war evacuation on cardiovascular health was studied in evacuees and non-evacuees. War evacuees had higher systolic blood pressure than non-evacuees, and the longer the separation from their biological parents the more prevalence for hypertension (Alastalo et al., 2009). In a separate study to determine the prevalence of CVD-related treatment, war evacuation was associated with being treated for coronary heart disease before and after adjusting for low socioeconomic status (Alastalo et al., 2012). Of the deceased

participants, the mortality and morbidity rates were also higher in war evacuees than non-evacuees (Alastalo et al., 2012). These findings showed that as the number of ACEs increased so does the prevalence of smoking and the use of illicit drugs. It is important to note that ACEs increase the susceptibility for risky behaviors such as smoking, obesity, physical inactivity, and intense alcohol consumption in adolescents and young adults (Anda et al., 1999; Felitti et al., 1998; Fuller-Thomson, Roane & Brennenstuhl, 2016; Isohookana, Marttunen, Hakko, Riipinen & Riala, 2016).

Sex differences exist in blood pressure across the life span and are attributed to differences in life expectancy, obesity, and sex hormones (Sandberg & Ji, 2012). Studies of ACEs also indicate sex differences in hypertension risk associated with various types of childhood adversity. Findings from a study of young adults who were exposed to ACEs (neglect, sexual abuse, or household dysfunction) prior to 6th grade (age 11) suggests sex differences in prevalence of hypertension may depend on the type of childhood stress (Suglia, Clark, Boynton-Jarrett, Kressin & Koenen, 2014). In this cohort, sexual abuse was the only factor associated with significantly higher prevalence of hypertension in women even when adjusted for socio-demographic factors such as sex, race, age, and education level compared to women without these ACEs. In contrast, though neglect, household dysfunction, and sexual abuse in men were associated with higher prevalence in hypertension, this association was not significantly different compared to men without childhood adversity (Suglia, Clark, Boynton-Jarrett, Kressin & Koenen, 2014). In the Helsinki cohort described above, the effect of childhood war evacuation on systolic blood pressure was significantly strongest in men (Alastalo et al., 2013) whereas no statistical significance was observed in women. Additionally, female

evacuees were on more antihypertensive medications compared to non-evacuees only when they were separated as toddlers. Evacuation for less than a year showed the most association with a higher systolic blood pressure only in females. Additionally, diastolic blood pressure in women evacuated for less than one year was higher than non-evacuees, whereas in men the highest diastolic blood pressure difference was in evacuees separated for longer than two years (Alastalo et al., 2013). Taken together, these studies show that childhood adversity negatively affects blood pressure throughout adult life span and the effect of ACE exposure at varying ages in childhood leads to different outcomes in men and women.

EVIDENCE FOR ASSOCIATIONS BETWEEN ACE EXPOSURES AND IMMUNE-MEDIATED OR VASOACTIVE PATHWAYS

Of the many mechanisms contributing to hypertension, inflammation and associated immune mediators and vasoactive factors are one of the many mechanisms contributing to hypertension and have been the most reported in studies with ACE exposure (Figure 2).

ACE Studies Linked to Inflammation and Immune Mediators in Children and Young Adults

The adaptive response to stress involves a hormonal cascade known as the hypothalamic-pituitary-adrenal (HPA) axis. Stress-induced activation of the HPA axis begins with the secretion of the hypothalamic hormone (corticotrophin-releasing hormone) that stimulates release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland into the systemic circulation, where it ultimately stimulates the

production and secretion of glucocorticoids (e.g., cortisol) from the adrenal cortex. When this normal response becomes dysregulated as a consequence of chronic stress, the resulting chronic exposure to corticosteroids can lead to several physiological changes that promote an increased risk of CVD including hypertension and inflammation.

When the normal HPA response becomes dysregulated as a consequence of chronic stress, the resulting chronic exposure to corticosteroids can lead to several physiological changes that promote an increased risk of CVD including hypertension and inflammation. One theory involving dysregulation of the HPA activity includes exhaustion of the system. Normal, physiological HPA activation elicits acute elevation of glucocorticoid levels, producing anti-inflammatory effects. In contrast, human subjects with unfavorable childhood circumstances display dysregulated cortisol secretion that correlates with increased levels of inflammatory markers. Bereaved children who experienced sudden parental death during the September 11, 2001 terrorist attacks were reported to exhibit higher cortisol salivary secretion compared to non-bereaved children, indicating dysregulated HPA function (Pfeffer, Altemus, Heo & Jiang, 2007) also found that subjects from low SES had elevated salivary cortisol secretion as well as displayed decreased activity of the glucocorticoid receptor responsible for the anti-inflammatory effects of cortisol and the negative feedback mechanism of the HPA axis. These findings were associated with elevated activation of NF-κB, a known pro-inflammatory transcription factor (Miller et al., 2009).

During the inflammatory process, cells of the immune system release cytokines and chemokines that modulate vascular function. Inflammation is necessary for wound healing and to ward off infectious diseases; however, if regulated inappropriately,

inflammatory pathways can lead to negative outcomes. Persistent release of inflammatory cytokines and chemokines leads to a chronic inflammatory condition albeit low-grade. Inflammation is well established as a mechanistic risk factor in developing CVD and CVD risk, especially in hypertension and vascular disease. In epidemiological studies outlined below, ACE exposures have been associated with elevated immune mediators suggesting that inflammation may be a possible mechanistic link between childhood adversity and hypertension risk in adulthood.

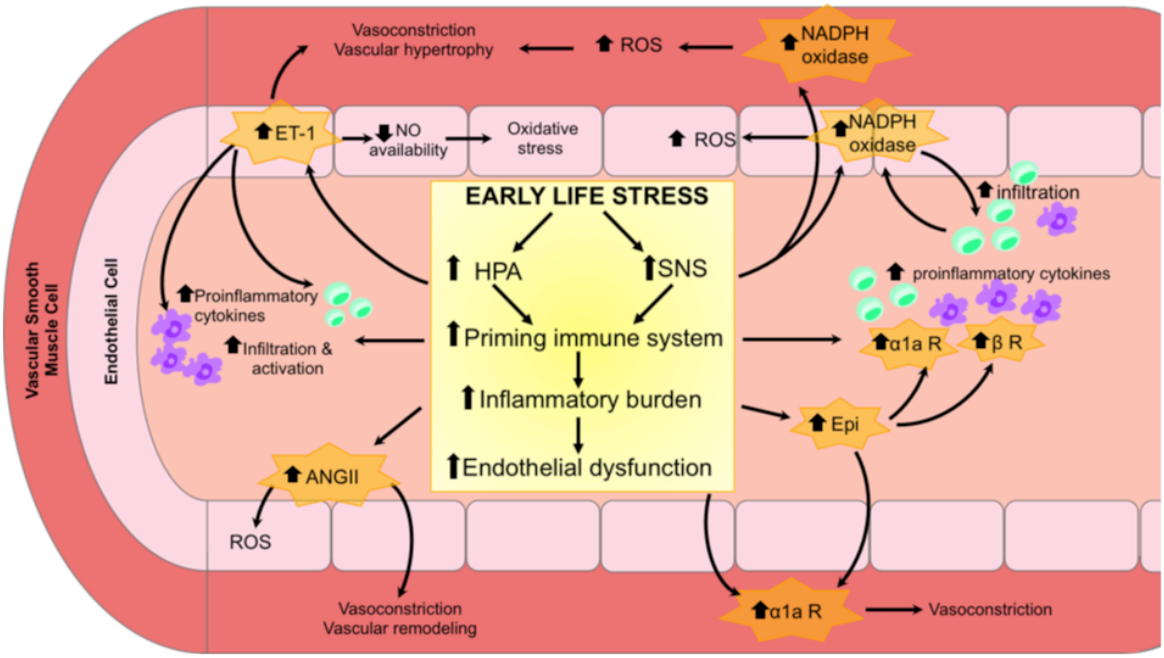


Figure 2. A schematic of potential molecular mechanisms involved in ACE exposure-induced immune mediated vascular dysfunction. Studies using rodent models of early life stress have indicated activation of inflammatory pathways that can contribute the development of hypertension and CVD. Additionally, increased activation and/or sensitivity of vasoactive pathways such as endothelin 1, renin angiotensin aldosterone system, oxidative stress, and sympathetic nervous system promote vascular dysfunction. Green cells: T cells; purple cells: Macrophages; R: receptor.

Inflammation has been reported in children exposed to childhood adversity prior to adolescence. Results from the Lifestyle Immune System Allergy (LISA) cohort study revealed that as early as 6 years of age, children who experienced adverse family events such as divorce and separation of parents show increased interleukin 4 (IL-4) compared to age-matched children without exposures to these experiences (Herberth et al., 2008). Authors in the Dunedin Multidisciplinary Heath and Development Study reported an association between childhood maltreatment and elevated levels of 3 common inflammatory markers associated with CVD: C-reactive protein (CRP), fibrinogen, and white blood cell count (Danese, Pariante, Caspi, Taylor & Poulton, 2007). In addition, a composite factor score of the summation of inflammatory markers is associated with childhood maltreatment (Danese, Pariante, Caspi, Taylor & Poulton, 2007). Limited parental education and low socioeconomic status during early life were also associated with increased levels of inflammatory markers. Women whose parents were less educated were positively associated with higher levels of CRP (Phillips, Marsland, Flory, Muldoon, Cohen & Manuck, 2009). IL-6 production was higher in cultured peripheral blood mononuclear cells (PBMCs) from subjects raised in a low socioeconomic environment compared to subjects raised in a high socioeconomic environment (Miller et al., 2009). In a meta-analysis study, childhood trauma was significantly associated to elevated inflammatory markers in adulthood, especially TNF-α and CRP (Baumeister, Akhtar, Ciufolini, Pariante & Mondelli, 2016). Other studies show increases in pro-inflammatory gene expression and inflammatory cluster membership using statistical modeling in adult subjects exposed to early life adversity (Ehrlich, Ross, Chen & Miller, 2016; Schwaiger et al., 2016).

While most studies have only measured the circulating levels of pro-inflammatory mediators in subjects exposed to childhood adversity, Elwenspoek et al. assessed T cell activation status in young adults with a history of childhood adversity (Elwenspoek et al., 2017). T cells that have not encountered any antigen (naïve or resting T cells) are activated by three main signals. First, cytotoxic or suppressor T cell ($CD8^+$ cells) and helper T cell ($CD4^+$ cells) recognize and interact with antigens that are present on major histocompatibility complex class I (MHC-I) and MHC-II molecules, respectively, being expressed on antigen presenting cells (APCs). Second, co-stimulatory molecules on T cells and antigen presenting cells (APCs) induce full T cell activation and proliferation. Lastly, the activated T cells produce specific cytokines further exaggerating and activating T cell proliferation and stimulation of cytokine production from other nearby immune cells (Goral, 2011). HLA-DR^+ (a human leukocyte antigen) is expressed during the process of T cell activation and CD25 (the alpha chain of IL-2 receptor) is expressed after the T cell receptor binding to antigens on MHC molecules. Stressful events stimulate T cell activation and increased expression of HLA-DR^+ on T cells (Elwenspoek et al., 2017). These authors reported that expression of HLA-DR^+ was significantly higher in $CD4^+$ cells and $CD8^+$ cells in adults with prior ACE exposures. In addition, $CD25^+$ cells were a significantly higher population of the $CD8^+$ cells with a similar trend in $CD4^+$ cells in adults with prior ACEs (Elwenspoek et al., 2017). Lemieux et has previously reported that females with post-traumatic stress disorder who experienced childhood abuse had significantly higher percentages of $CD8^+CD45RA^+$ cells compared to females with no PTSD diagnosis (Lemieux, Coe & Carnes, 2008). In the literature, T cells expressing these markers are classified as naïve or terminally differentiated effector

memory cells depending on the co-expression of other surface markers (Larbi & Fulop, 2014; van den Broek, Borghans & van Wijk, 2018). Unfortunately, in this study of females with post-traumatic stress disorder, co-expression of other surface markers was not evaluated. Collectively, findings from these studies suggest that ACEs enhance the percentage of T cells, induced or primed T cell activation, and a pro-inflammatory state.

ACE Studies Linked to Inflammation and Immune Mediators in Older Adults

Studies on the influence of childhood adversity on inflammation in adults over 60 years old is sparse mainly because the results are compounded by existing chronic medical conditions that may contribute to the observed elevated inflammation in those patients. The International Mobility In Aging Study (IMIAS) examined the effect of childhood adversity on CRP in adults 65-75 years old living in Brazil, Canada, and Columbia. The results indicated that social adversity was associated with higher CRP in the Canadian population than populations from Brazil and Columbia. Childhood social adversity, not childhood economic adversity or poor health during childhood, was an independent predictor of chronic inflammation in old age in the Canadian sample (Li et al., 2015). Another study in adults older than 60 years of age who experienced childhood physical, emotional or sexual abuse revealed that any form of childhood abuse was associated with elevated levels of IL-6 and adults who experienced more than one form of abuse had significantly higher levels of IL-6 (Kiecolt-Glaser, Gouin, Weng, Malarkey, Beversdorf & Glaser, 2011). Overall, an association remains between childhood adversity and pro-inflammatory markers in adults of all ages.

ACE Studies on Sex Differences Linked to Inflammation and/or Immune Mediators

Sex differences exist with inflammation in subjects with childhood adversity. Children raised in low socioeconomic environment are at risk for developing CVD risks in adult life. As one study revealed, an increase in adiposity may be a factor contributing to greater CVD in adults raised in low socioeconomic background (Shrewsbury & Wardle, 2008). The Avon Longitudinal Study of Parents and Children examined whether associations existed between maternal education and cardiovascular risk factors including CRP and IL-6 in boys and girls (Howe et al., 2010). Results from their study showed that girls tended to have more fat mass than boys and higher levels of CRP (Howe et al., 2010); however, adiposity partially explained the increase in CVD risks. Conversely, having a greater psychological resource such as being optimistic, high self-esteem, and perceived control of one's life was associated with lower IL-6 but not CRP in adult men who were raised in a low socioeconomic environment according to the MIDUS study (Elliot & Chapman, 2016). No associations were observed in women. All of these studies highlight the complexity of sex differences of immune responses in children and adults who experienced childhood adversity and the need for more in-depth studies in order to fully understand the mechanistic pathways mediating these sex differences.

ACE Studies Linked to SNS, Inflammation and Immune Mediators

Activation of the sympathetic nervous system (SNS) triggers inflammation and immune system activation (Danese & McEwen, 2012). ACEs occur in children at critical developmental periods when the brain is susceptible to environmental changes. The amygdala is important in emotional development, survival instincts, and memory and has shown to be involved in stress responses and activation of the SNS. Increased amygdala

activity has been reported in institutionalized children (children in foster care), suggesting exaggerated SNS activity (Tottenham, Hare, Millner, Gilhooly, Zevin & Casey, 2011). Kuras et al determined SNS reactivity in healthy individuals with and without childhood adversity and found that healthy subjects with childhood adversity had a higher salivary alpha-amylase, a marker of SNS reactivity, in response to acute psychosocial stress induced by the Trier Social Stress Test compared to individuals without childhood adversity (Kuras et al., 2017). Data from the Coronary Artery Risk Development in Young Adults (CARDIA) study revealed that urinary catecholamine levels were higher in low socioeconomic adult individuals independent of race, gender, and age. β-adrenergic receptors are targets for catecholamines released during SNS activation and are highly expressed on various immune cells, including T cells and monocytes. A study using populations from a low and high socioeconomic status found that circulating PBMCs from individuals of low socioeconomic status (SES) had increased expression of specific pro-inflammatory genes (TNF-α, IL-8, and IL-1β) compared to individuals from higher socioeconomic background. These changes in expression were found to be associated with higher activity of the β-adrenergic transcription factor cAMP response element binding protein (CREB) in circulating PBMCs as well as increased circulating catecholamines in individuals of low socioeconomic status (Powell et al., 2013). An additional study using data from the Midlife in the United States (MIDUS) cohort found that ACEs were a strong predictor of inflammation associated with higher levels of urinary norepinephrine excretion, an index of SNS activity (Hostinar, Lachman, Mroczek, Seeman & Miller, 2015). These findings indicate that the link between

inflammation and upregulation of SNS activity in individuals exposed to ACEs may be a contributing factor to the increased risk of inflammatory disease, CVD, and CVD risks.

ACE Studies Linked to Vasoactive Pathways

Endothelin

Endothelin 1 (ET-1) is a potent vasoconstrictor peptide and a known immune mediator produced mainly by endothelial cells (Davenport et al., 2016; Vuurmans, Boer & Koomans, 2003). Su et al. explored whether there is an association between ACE exposure and plasma ET-1 levels, total peripheral resistance, blood pressure, pulse wave velocity, and cardiac output index in adolescent and young adults (Su et al., 2014). Indeed, there were significant associations between two or more ACEs and plasma ET-1, as well as diastolic blood pressure, total peripheral resistance, and pulse wave velocity independent of age, race, sex, body mass index, and childhood socioeconomic status. Another corresponding study found higher circulating ET-1 levels in adults who grew up in a lower SES population compared to a higher SES independent of ethnicity, gender, smoking, and blood pressure (factors known to effect levels of inflammation) (Hong, Nelesen, Krohn, Mills & Dimsdale, 2006). Collectively, these studies suggest ET-1 is one vasoactive pathway through which exposure to ACEs may promote CVD risk (Su et al., 2014).

Leptin

Leptin is a hormone produced by adipocytes regulating food intake and energy balance. The action of leptin on the hypothalamus decreases appetite and increases energy expenditure. Any defect in the leptin gene results in obesity and cardiovascular

disease (Beltowski, 2006). Earlier studies in humans showed that leptin stimulates the SNS (Eikelis, Schlaich, Aggarwal, Kaye & Esler, 2003) and may play a role in microcirculation via nitric oxide dependent pathway (Tsuda, Kimura & Nishio, 2002). Hypertensive patients have significantly elevated plasma leptin levels (Agata et al., 1997). Positive correlations between blood pressure and plasma leptin concentration have been reported in both normotensive and hypertensive individuals (Kazumi, Kawaguchi, Katoh, Iwahashi & Yoshino, 1999; Schutte, Huisman, Schutte & Malan, 2005). By the same token, an association exists between leptin and blood pressure in individuals with prior ACEs. In a study by Cromwell et al., individuals from diverse socioeconomic economic backgrounds were recruited in order to determine the effect of ACEs on physical health (Crowell et al., 2016). Pathway analysis models were used to estimate the magnitude and significance of the hypothesized links between ACEs and leptin, blood pressure, dietary quality and central obesity. Their findings showed that ACE exposures was associated with elevated circulating leptin, which was subsequently associated with increased blood pressure (Crowell et al., 2016). These studies together suggest that leptin may be a mechanistic link with ACEs and CVD risk.

RODENT MODELS OF EARLY LIFE STRESS-INDUCED HYPERTENSION RISK

Epidemiological studies in humans are confounded by a plethora of variables such as diet, lifestyle, underlying illness, age, and sex that can influence the direct effects of childhood adversity on CVD and hypertension risk even though rigorous statistical analyses are used to control for such variables. Animal models aid in deciphering

mechanistic links between early life stress and outcomes of CVD progression and risk factors.

Cardiovascular health in adult life may be reprogrammed by interfering with normal offspring development during prenatal and postnatal stages of life. Prenatal stress or fetal programming proposes that changes in maternal environment caused by maternal genes, undernutrition, overnutrition, placental insufficiency, or behavior such as maternal smoking and drug abuse in humans during pregnancy results in cardiovascular disease, metabolic syndrome, and diabetes in adult life (Hocher, 2014). On the contrary, postnatal re-programming involves inducing stressful events to the offspring during the first several weeks of life. Various animal models have been used to elucidate the effects of ELS on health and disease progression. Historically, mouse and rats are the most commonly used animal model in cardiovascular disease research. The use of small rodents such as rats and mice have provided valuable input in the pathophysiology of various diseases, including CVD. However, there are limitations using these animal models in the study of CVD. For instance, in studying heart disease, the smaller hearts and body weights of animals highlight the difference in their physiology when compared to humans (Milani-Nejad & Janssen, 2014; Recchia & Lionetti, 2007). Additionally, small animal models are phylogenetically distant from humans and may have variable responses to pharmaceutics between species(Camacho, Fan, Liu & He, 2016; Recchia & Lionetti, 2007). Due to these limitations, pathophysiology of disease is not fully translatable to humans. Despite these setbacks, utilization of relevant small rodent animal models is still an important necessity to unravel pathological mechanisms involved in the development of any human disease. Mice and rats are the most commonly used *in vivo*

models in studies of physiological functions due to their high availability, cost effectiveness, and common use in research compared to other species. Additionally, small rodent models have a higher availability of multiple knockout and transgenic models, especially in mice. Since mouse and rat models are the most commonly used animal models in studying the pathophysiology of CVDs, we solely focus on postnatal rodent models for studying the role of ELS in CVD and hypertension risk.

Maternal Separation (MatSep) Rat Model of ELS

MatSep in rats is the most common postnatal model for studying the effects of ELS. Rodent pups are completely dependent on their mothers for food, warmth, and grooming/licking during the first two weeks of life. This interaction between mother and offspring is important for developing proper emotional, behavioral, cognitive, and physiological functions (Vetulani, 2013). In rats, MatSep involves separating pups from the dam during the stress hypo-responsive period (Levine, 1967; Tata, 2012). The maternal-offspring relationship during early development reduces the HPA axis response to stress (Liu et al., 1977). Any prolonged interference with the maternal-offspring relationship induces HPA axis hyper-responsiveness, thereby increasing stress hormones such as catecholamines and glucocorticoids/cortisol (Ladd, Huot, Thrivikraman, Nemeroff, Michael J. Meaney & Plotsky, 2000; McEwen, 2003; Pfeffer, Altemus, Heo & Jiang, 2007).

Several studies have determined that the timing and duration of MatSep is important for studying long-term consequences that persist until adulthood (Vetulani, 2013). MatSep lasting 3 hours a day from postnatal day (PD) 2 to PD 14 generally leads to HPA hyper-responsiveness and is characterized by permanent depression and anxiety-

like behaviors with long lasting psychological and behavioral consequences (Daniels, Pietersen, Cartens & Stein, 2004; Tata, 2012; Vetulani, 2013). Even though MatSep has been extensively described as a model for studying behavioral and emotional disorders, in recent years it is now known to have long lasting immune and cardiovascular consequences as well.

Apart from timing, there are strain specific differences in anxiety vulnerability in rodents. A study was conducted in the inbred Wistar-Kyoto (WKY) and Sprague-Dawley rats to determine strain susceptibility to hypervigilance as a measure of anxiety vulnerability assessed using the open field test (McAuley, Stewart, Webber, Cromwell, Servatius & Pang, 2009). Compared to Sprague Dawley rats, WKY rats stayed longer in the middle of the circle drawn on an open floor, made fewer crossings over lines drawn from the center of the circles, and reared less -- all indications of anxiety-like behaviors. These findings confirmed the WKY rat strain to be a good model for studying anxiety behaviors (McAuley, Stewart, Webber, Cromwell, Servatius & Pang, 2009). Another study compared WKY rat behavioral responses, measured by the forced swim test and social interaction, to Sprague-Dawley rats, Wistar rats, and Spontaneously Hypertensive rats (SHR). Only WKY rats showed increased immobility on forced swim test and increased social avoidance, which are an indication of anxiety and depressive like behaviors that have a strong genetic influence on the observed phenotype (Nam, Clinton, Jackson & Kerman, 2014). Rana and colleagues reported that MatSep in WKY rats decreased anxiety- and depressive-like behaviors in adulthood, but had an opposite effect in Wistar rats, leading to enhanced anxiety-like behaviors in adults. These findings are consistent with the predictive adaptive response, which suggests that ELS exposure can

confer adaptive value in later life within certain individuals (Rana, Pugh, Jackson, Clinton & Kerman, 2015).

Maternal Separation with Early Weaning (MSEW) Mouse Model of ELS

Strain differences in the consequences of maternal separation have also been observed in mice. Differences in anxiety-like behaviors were determined in nine mouse strains (BALB/cByJIco, C57BL/6JIco, C3H/HeOuJIco, CBA/JIco, DBA/2JIco, NZB/Ola/Hsd, SJL/J, NMRI, and Swiss). C57BL/6JIco, DBA/2JIco, and NZB/Ola/Hsd mice displayed high levels of anxiety-like behavior compared to the other strains (Griebel, Belzung, Perrault & Sanger, 2000). Thus, different early life stress protocols of varying durations of maternal separation with or without early weaning have been used depending on the mouse strain. One of the most robust ELS protocols for mouse models is maternal separation with early weaning (MSEW). MSEW closely depicts the behavioral dysfunction, such as depression and anxiety, often observed in humans with a history of ACEs (George, Bordner, Elwafi & Simen, 2010). MSEW involves separating the pups from the dams for 4 hours during PD 2-5, then 8 hours during PD 6-16, and ends with early weaning on PD 17; control litters are left undisturbed and normally weaned on PD21. George et al found that C57Bl/6J and DBA/2J mouse strains differed in behavioral tests indicating increased anxiety-like behaviors (but not body weight or metabolites) more than two months after the MSEW protocol, indicating disparities in the HPA axis. In comparison, other protocols involving maternal separation only deviate from the MSEW protocol with hours of maternal separation and/or age. Thus, understanding strain specific differences in depressive and anxiety-like behaviors in rodents is important in

choosing the appropriate model for studying ELS effects on immune mediators and cardiovascular outcomes.

Animal studies gives a more in-depth insight of the interactions between the HPA and HPG axes. During sexual development and maturation, sex differences in the function of the HPA axis is mainly due to sex hormones. In females, estrogen and progesterone levels fluctuates from puberty through adult life. A high estrogen level corresponds to high CRH, ACTH, and corticosterone an indication that estrogen stimulates the HPA axis (Walker, Francis, Cabassa & Kuhn, 2000) through increased CRH stimulation (Mastorakos, Pavlatou & Mizamtsidi, 2006). On the contrary, some studies have reported an inhibitory effect of estrogen on the HPA axis during times of stress. During restraint stress, administration of low dose estradiol reduced ACTH levels in ovariectomized rats (Young, Altemus, Parkinson & Shastry, 2001). Additionally, progesterone and estradiol given in combination produced an inhibitory effect on ACTH in response to restrained stress (Young, Altemus, Parkinson & Shastry, 2001). Male sex hormones remain constant during puberty and in adult life although it has been reported that there is increase in testosterone pre-puberty (Oyola & Handa, 2017). In another acute stress study, ACTH in pre-pubertal males were higher than in adult males (Romeo et al., 2006). Corticosterone levels are equally higher in prepubertal and adult males during acute stress; however, stress induced increase in corticosterone level is shown to be higher in prepubertal males than in adult males (Romeo et al., 2006). The authors also found that free corticosterone was significantly higher in prepubertal males immediately after the stressor compared to adults, this difference was reversed in 45 minutes after

removal of the stressor (Romeo et al., 2006). These findings support the influence of sex hormones in HPA axis response to stress.

PRO-INFLAMMATORY MEDIATORS AND VASOACTIVE FACTORS AS MECHANISMS OF ELS-INDUCED HYPERTENSION RISK

Over the past 10-15 years, epidemiological studies have associated ACEs with increases in immune mediators as well as vasoactive factors. As a modulator of cardiovascular diseases, inflammation is induced by disruption in tissue homeostasis, mediated by the innate and adaptive immune systems. The innate immune system is the first line of defense against invading pathogens and tissue injury via recognition of pathogen-associated molecular recognition patterns (PAMPs) and damage-associated molecular recognition patterns (DAMPs). Both PAMPs and DAMPs bind specific receptors such as Toll-like receptors (TLRs) that are expressed on immune cells including neutrophils, dendritic cells, and macrophages (Tang, Kang, Coyne, Zeh & Lotze, 2012). Conversely, the adaptive immune system relies on antigen presentation from innate immune cells to become activated. Activation of both the innate and adaptive immune systems produce pro-inflammatory cytokines that can promote tissue injury (Yu et al., 2017) Figure 2. Inflammatory pathways can be mediated by autonomic and neuroendocrine mechanisms. Immune cells, such as lymphocytes and phagocytic immune cells, produce catecholamines and express adrenergic receptors. Catecholamines produced by lymphocytes act in an autocrine and paracrine manner by increasing Th1 and Th2 cytokine production and modulate the actions of nearby cells (Flierl, Rittirsch, Huber-Lang, Sarma & Ward, 2008). Similar to humans who experience ACEs, ELS in

mice induces HPA axis dysregulation that ultimately leads to increased secretion of glucocorticoids and catecholamines. Inflammatory pathways trigger elevated glucocorticoid and catecholamine secretion in response to stress (Bellinger et al., 2008; Busillo, Azzam & Cidlowski, 2011; Elenkov, 2008; Huang et al., 2012; Zhou, Xu & Jiang, 2008). These triggered inflammatory pathways have been shown to have detrimental effects on cardiovascular function through actions of vasoactive mediators such as ET-1 as well as changes in autonomic and neuroendocrine pathways (Kahler et al., 2001; Liu, Wang & Jiang, 2017; Silverman & Sternberg, 2012) Figure 2.

ELS Studies and Hypertension Risk Linked to Pro-inflammatory Mediators

Studies have documented cardiovascular changes and elevated inflammatory mediators in ELS rodent models. Imposition of ELS in Sprague Dawley rats by MatSep exposure during the first 2 weeks of life or in Wistar rats via early weaning induces hypertensive states at both adolescent and adult ages (Franco et al., 2013; Genest, Gulemetova, Laforest, Drolet & Kinkead, 2004; Reho & Fisher, 2015). In borderline hypertensive rats, Sanders et al. reported that MatSep for 3 hours per day on PD 1-14 induced enhanced stress activity. When exposed to a second hit of stress during adulthood, MatSep rats had significantly higher heart rate compared to controls (Sanders & Anticevic, 2007). In MSEW mice, imposing a high fat diet as a secondary stressor increased systolic blood pressure and MAP (Murphy, Herald, Leachman, Villasante Tezanos, Cohn & Loria, 2018). Various strains of mice and rats exhibit ELS-induced changes in inflammation. Maternal separation alone in C57Bl/6 mice has been shown to increase phagocytosis in circulating neutrophils, a process mediated by cytokines (Pinheiro, Ferraz-de-Paula, Ribeiro, Sakai, Bernardi & Palermo-Neto, 2011). MatSep

rodents also develop autoimmune diseases. In a study examining parameters of irritable bowel syndrome, MatSep in mice induced pro-inflammatory status of T cells as indicated by activated splenic T cells and increased IFN-γ secretion from splenic T cells after antiCD3/CD28 stimulation (Riba et al., 2018). Alterations in immune cell populations and functions have also been observed in various rodent strains exposed to MatSep including Sprague Dawley, Wistar, and FSL rat strains as well as C3H/H3N and NMRI mouse strains (Carboni et al., 2010; Pinheiro, Ferraz-de-Paula, Ribeiro, Sakai, Bernardi & Palermo-Neto, 2011; Riba et al., 2018; Roque, Mesquita, Palha, Sousa & Correia-Neves, 2014). Additionally, MatSep in rats and mice increases circulating levels of inflammatory cytokines including IL-1β and IL-6 (Carboni et al., 2010; Hohmann, Odebode, Naidu & Koban, 2017; Roque, Ochoa-Zarzosa & Torner, 2016; Wang et al., 2017; Wieck, Andersen & Brenhouse, 2013). Collectively, these studies show the importance of MatSep-induced immunological changes in disease development. Despite these observations, few investigations have linked inflammatory mediators and hypertension associated with ELS.

In a separate study by our group, chronic angiotensin II (AngII)-induced hypertension led to significantly exaggerated blood pressure along with increased macrophages and T cells in the endothelium and perivascular adventitia of the aorta (Loria, Pollock & Pollock, 2010) as well as elevated numbers of T cells in kidneys from MatSep rats compared to normally-reared controls (Loria, Yamamoto, Pollock & Pollock, 2013). Overall, findings from these studies are significant in several ways. First, the kidney is the major regulator of long-term blood pressure. Therefore, inflammation in the kidneys, as shown by several studies, would lead to renal injury and improper blood

pressure regulation resulting in higher blood pressure observed in MatSep rats. Second, the heightened response of inflammatory genes to LPS, macrophages, T cells, and blood pressure to chronic AngII treatment further supports the epidemiological findings that prior exposure to early life adversity reprograms or primes immune and cardiovascular processes. Third, the presence of a pro-inflammatory phenotype in MatSep kidneys compared to control kidneys without the observation of blood pressure differences in both groups is an indication that of the presence of a "primed" pro-inflammatory phenotype that may mediate the exaggerated blood pressure response when MatSep rats are exposed to AngII or a "second hit".

Pro-hypertensive Immunological Memory

Though it has not been thoroughly examined in ELS animal models, immunological memory has been shown to play a central role in the development of hypertension induced by a "second hit". The capability of the immune system to provide long time protection against various types of infections relies on immunological memory, an essential role of the adaptive immune response. The long-lived immune memory cells are characterized by increased specificity and efficacy of a secondary immunological response (Danese & J Lewis, 2017; Dowling & Levy, 2014; Ygberg & Nilsson, 2012). Transformation of immune memory T-cells is a process that requires T-cells and antigen presenting cells (APCs) interaction through the T-cell surface marker CD27 and activated APCs surface marker CD70 (Denoeud J, 2011; Keller, Schildknecht, Xiao, Van den Broek & Borst, 2008; Sallusto, Geginat & Lanzavecchia, 2004). In the T-cell population, a subset of memory T-cells that differentially express surface markers $CD44^{hi}/CD62L^{hi}/CCCR7^{+}$, known as central memory T cells (T_{CM}), eventually reside in

secondary lymphoid organs that readily proliferate in response to antigenic stimulation and plays a role in reactive memory (Campbell et al., 1998; Forster et al., 1999; Lanzavecchia & Sallusto, 2000; Sallusto, Geginat & Lanzavecchia, 2004). Protective memory is mediated by effector memory T cells (T_{EM}), another subset population of memory T-cells (Lanzavecchia & Sallusto, 2000). T_{EM} cells differentially express $CD44^{hi}/CD62L^{lo}/CCCR7^{-}$ surface markers and produce various chemokine receptors and adhesion molecules that allows them to invade sites of inflammation (Campbell et al., 1998; Forster et al., 1999; Lanzavecchia & Sallusto, 2000).

In the development of hypertension, Itani et al studied the various memory T-cell populations in several models of repeated hypertensive stimuli (Itani et al., 2016). CD4+ and CD8+ T_{EM} cell populations in kidneys and bone marrow as well as CD70 expression in splenic dendritic cells and macrophages from mice treated with LNAME followed by a high salt diet were significantly higher compared to control mice, mice treated with LNAME only, and mice treated with high salt diet alone. Moreover, when L-NAME/high salt treated mice were exposed to a second additional high salt treatment after a recovery period, salt sensitive hypertension sustained many months after administering L-NAME and produced an even greater increase in the renal T_{EM} cells compared to mice treated with L-NAME followed by only 1 exposure of high salt diet. Similar results were also observed when mice were treated with a high dose of Ang II followed by a low dose of Ang II after a recovery period. Furthermore, exposure to high diet salt in normal control mice that received an adoptive transfer of T_{EM} cells from L-NAME/high salt treated mice induced T_{EM} cell expansion in the bone marrow as well as in the kidneys.

Several studies using IFN-γ KO mice and/or IL-17A KO mice have shown elevated IFN-γ and IL-17A production contributes to altered tubular function promoting salt sensitivity as well as mediates the hypertension response to Ang II and aldosterone infusions and the resulting end organ damage, including renal damage and vascular dysfunction (Garcia et al., 2012; Itani et al., 2016; Kamat et al., 2015; Marko et al., 2012; Nguyen, Chiasson, Chatterjee, Kopriva, Young & Mitchell, 2013; Oh et al., 2002; Satou, Miyata, Gonzalez-Villalobos, Ingelfinger, Navar & Kobori, 2012; Trott et al., 2014; Wu et al., 2014). Additionally, T_{EM} cells are the major source of IFN-γ and IL-17A in the kidneys (Itani et al., 2016).

The immune system development begins during the prenatal stage; however, both innate and adaptive immunity are not fully developed until adolescent years (Dowling & Levy, 2014; Ygberg & Nilsson, 2012). Hence, early life exposure to various external stimuli such as repeated antigenic response can affect the development of the immune system (Danese & J Lewis, 2017). Thus, repeated exposures to ELS altering the maturation of the immune system by priming immunological memory cells may be a plausible mechanism of ELS-induced increased hypertension risk.

ELS-induced Hypertension Linked to Vasoactive Factors

Catecholamines

Stress responses stimulate production and release of catecholamines, specifically norepinephrine and epinephrine. Heightened SNS activity can contribute to physiological changes that promote the development of hypertension. For example, Reho and Fisher demonstrated that MatSep in Sprague Dawley rats was associated with elevated mRNA levels of CPI-17 (a protein that inhibits smooth muscle myosin phosphatase, thereby

promoting contraction) and α_{1a}-adrenergic receptor, as well as increased mesenteric artery hypercontractility at PD 21. This affect was reversed when MatSep Sprague Dawley rats were treated with an α-adrenergic receptor antagonist. By PD 35, although MatSep induced mesenteric artery hypercontractility had dissipated, elevated α_1-adrenergic receptor mRNA expression persisted. Moreover, MAP trajectory between PD 21 and 35 was higher in MatSep rats compared to controls (Reho & Fisher, 2015). Data from a separate study investigating ventilatory parameters suggest elevated MAP induced by MatSep in male Sprague Dawley rats may be due to greater sympathetic outflow as suggested by enhanced carotid body function (Genest, Gulemetova, Laforest, Drolet & Kinkead, 2004). Additionally, elevated norepinephrine levels have been observed in various organs, specifically in the renal cortex, outer medulla and inner medulla, as well as the left ventricle, spleen and adrenal glands from rats exposed to MatSep when compared to normally-reared controls (Loria, Brands, Pollock & Pollock, 2013). Elevated levels of adrenaline and noradrenaline have also been reported in the gut and brain of mice and rats exposed to ELS (Liu, Caldji, Sharma, Plotsky & Meaney, 2000; Moya-Perez, Perez-Villalba, Benitez-Paez, Campillo & Sanz, 2017). Acute norepinephrine administration in conscious MatSep rats led to a significant increase in blood pressure compared to control rats. Further, ganglionic blockade prior to the acute norepinephrine treatment significantly decreased the rise in blood pressure in MatSep rats over that in control rats, an indication of higher sympathetic tone in the MatSep rats (Loria, Brands, Pollock & Pollock, 2013). Recently, it was reported that the renal vasculature in MatSep rats had a lower density of α-adrenergic receptors and a diminished vasoconstrictor response when the vessels were stimulated with an α-adrenergic receptor agonist

compared to control rats (Loria & Osborn, 2017). These findings suggest that exposure to ELS prompts adaptive processes in the renal vasculature, down-regulating adrenoceptor activity in the presence of MatSep-induced elevations in norepinephrine levels; however, these studies did not assess norepinephrine levels or adrenergic receptor expression in renal immune cells. The heightened norepinephrine levels in kidneys from MatSep rats may be derived from renal immune cells leading to the consequent pro-inflammatory state.

Catecholamines and SNS activation have been shown to play a role in disease phenotype observed when with other types of stress inductions in rodents. For example, the analysis of the genetic profile in mice that have endured social stress (a 2-hour exposure to an aggressive male intruder mouse) showed up-regulation of a pro-inflammatory phenotype of splenic monocytes mediated by elevated β-adrenergic receptor expression in monocytes (Powell et al., 2013). Although these studies support the hypothesis that ELS stress is a possible stimulator of pro-inflammatory pathways, further investigation is needed to fully understand the exact role of catecholamines and SNS activation in immune cells and their contribution to ELS induced hypertension and CVD risk in rodents.

Glucocorticoids

Similar to humans, ELS in rodents have been shown to induce dysregulation of the HPA axis that is associated with changes of various pathways that promote disease. Adult Sprague Dawley rats exposed to neonatal MatSep display elevated arterial pressure associated with higher levels of plasma ACTH and cortisol, as well as elevated *c-fos* mRNA expression in the paraventricular nucleus of the hypothalamus compared to

controls (Powell et al., 2013). Alterations of cortisone levels because of dysregulated HPA axis have been associated with pro-inflammatory pathways and SNS activation in rodents and can potentially promote the development of hypertension. In a study of Long-Evans hooded rats, elevated circulating ACTH led to increased norepinephrine in the hypothalamus (Liu, Caldji, Sharma, Plotsky & Meaney, 2000). MatSep-induced changes in T cell population in Wistar rats were associated with elevated circulating cortisone(Roque, Mesquita, Palha, Sousa & Correia-Neves, 2014). Alterations in cortisol levels after MatSep in adult mice has been associated with increased circulating neutrophil activity. Neuroinflammatory regulation of the HPA axis has also been shown to occur in rodents in response to stress. MatSep in both mice and rats have led to an increase in pro-inflammatory cytokines IL-1β and IL-6, as well as a decrease in the anti-inflammatory cytokine IL-10 (Amini-Khoei et al., 2017; Roque, Ochoa-Zarzosa & Torner, 2016; Wang et al., 2017). IL-1β has been shown to promote persistent HPA axis activation (Wang et al., 2017).

Reactive oxygen species (ROS)

ROS play an important role in vascular function and inflammation associated with the development of hypertension. Various sources of ROS production include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, mitochondrial-derived ROS, xanthine oxidase-derived ROS, and uncoupled nitric oxide synthase (NOS) (Mueller, Laude, McNally & Harrison, 2005). In the vasculature, hypertensive stimuli such as ANGII and catecholamines are associated with upregulation and increased activity of the NADPH oxidase pathway, the predominant source of ROS in the development of hypertension (Griendling, Minieri, Ollerenshaw & Alexander, 1994;

Landmesser et al., 2002; Landmesser et al., 2003; Matsuno et al., 2005; Mohazzab, Kaminski & Wolin, 1994; Pagano, Ito, Tornheim, Gallop, Tauber & Cohen, 1995; Rajagopalan et al., 1996). Another major source of ROS in vessels is uncoupled NOS (Landmesser et al., 2003; Matsuno et al., 2005). Additionally, several *in vitro* and *in vivo* studies have revealed that vascular changes such as vasoconstriction, vascular smooth muscle hypertrophy, and possibly increased vascular stiffness as a result of increased vascular collagen production are induced by NADPH oxidase or uncoupled nitric oxide synthase (NOS) mediated ROS production (Patel, Cardneau, Colles & Graham, 2006; Pu, Neves, Virdis, Touyz & Schiffrin, 2003; Taddei, Virdis, Ghiadoni, Magagna & Salvetti, 1998; Virdis, Neves, Amiri, Touyz & Schiffrin, 2004; Weber et al., 2005; Zafari et al., 1998).

Phagocytic cells and lymphocytes also express NADPH oxidase (Mittal, Siddiqui, Tran, Reddy & Malik, 2014). Chronic ROS production can modulate pro-inflammatory cytokine production as well as promote increased activation of inflammatory cells. For example, studies have shown NADPH oxidase mediated ROS production results in activation of NFκB and heat shock proteins which ultimately leads to expression of adhesion molecules such as ICAM1 (Gorman, Heavey, Creagh, Cotter & Samali, 1999; Kol, Bourcier, Lichtman & Libby, 1999; Manning et al., 1995; Polla & Cossarizza, 1996; Sen & Packer, 1996). Studies have also reported that vascular ROS production via NADPH oxidase are causally linked to infiltration of various immune cells induced by AngII and hypertension (Bayraktutan, Draper, Lang & Shah, 1998; Griendling, Minieri, Ollerenshaw & Alexander, 1994; Pagano, Chanock, Siwik, Colucci & Clark, 1998). For example, one study has shown renal and vascular dysfunction that are known to

contribute to the development of hypertension may be dependent on NADPH oxidase induced activation of T cells (Hoch et al., 2009). Another *in vivo* study using Ang II infused mice determined that dendritic cells from hypertensive mice produce extensive amounts of ROS (Kirabo et al., 2014). Furthermore, release of IL-6, IL-1β, and IL-23 from these activated dendritic cells promoted proliferation and an inflammatory phenotype of T cells. Another example from a study by Liu et al indicates macrophage infiltration induced by Ang II is mediated by vascular NADPH oxidase *in vivo* (Liu, Yang, Yang, Jankowski & Pagano, 2003).

Though several studies suggest ROS mediated inflammatory and vascular effects plays an important role in the development of hypertension, few studies have examined the pathophysiological role of ROS in ELS-induced CVD risk and associated hypertension. Our group showed that expression of genes encoding NADPH oxidase subunits *Nox2 and Nox4* are upregulated in aorta from mice exposed to MSEW, and that NADPH oxidase inhibition with apocynin improves MSEW-mediated endothelial dysfunction (Ho, Burch, Musall, Musall, Hyndman & Pollock, 2016). In contrast, although constrictor responses to acute AngII treatment are exaggerated in aorta from MatSep rats, this phenomenon is unaffected by treatment with apocynin or cell-permeable superoxide dismutase, suggesting that other ROS-dependent pathways may be involved (Loria, Kang, Pollock & Pollock, 2011). Neither MatSep nor MSEW studies have explored the effect of ROS on immune cells. Furthermore, MatSep and MSEW models show differences in ROS-mediated mechanisms of CVD, which may be a result of differences in immune regulation in both models.

In another type of ELS stress model, consequences of early weaning in Wistar rats included hypertension and higher oxidative stress indicated by increased plasma and liver TBARS and lower plasma superoxide dismutase. When treated with resveratrol, blood pressure and oxidative stress were lowered to levels comparable to that observed in control rats (Franco et al., 2013).

Nitric oxide (NO)

NO is an important signaling molecule during inflammation (Bogdan, 2015; MacMicking, Xie & Nathan, 1997). It is produced by many cells, including endothelial and immune cells, by the enzymatic action of nitric oxide synthase (NOS) (Bogdan, 2015; Bogdan, 2001; Darwiche et al., 2012; Moncada, Palmer & Higgs, 1989; Wink et al., 2011). The three NOS isoforms are NOS1, NOS2 and NOS3 (Bogdan, 2015; Forstermann & Kleinert, 1995; Forstermann & Sessa, 2012). Both NOS2 and 3 have been shown to be expressed in immune cells (Bogdan, 2015; MacMicking, Xie & Nathan, 1997). However, NOS2, the inducible form of NOS, plays a primary role in inflammation and activation of immune cells (Bogdan, 2015; Bogdan, 2001; Bogdan, 2011; Wink et al., 2011). During inflammation, circulating neutrophils and macrophages move to the site of inflammation producing NO (Adler et al., 2010; MacMicking, Xie & Nathan, 1997). In turn, the NO produced by these immune cells acts as an inflammatory mediator by signaling the influx of more immune cells to the inflammatory tissue (Bogdan, 2001; Bogdan, 2011; Martinelli, Gegg, Longbottom, Adamson, Turowski & Greenwood, 2009). However, overproduction of NO by immune cells can damage tissues as a result of superoxide production via NOS uncoupling (Dinh, Drummond, Sobey & Chrissobolis, 2014; McCall, Boughton-Smith, Palmer, Whittle & Moncada, 1989; Sharma, Al-Omran

& Parvathy, 2007). Hence, the action of NO may be pro- or anti-inflammatory depending on its concentration (Sharma, Al-Omran & Parvathy, 2007). In the study evaluating the role of SNS activation in ELS-induced alterations of vessel reactivity described above, MatSep-induced mesenteric arterial hypercontractility in Sprague Dawley rats was accompanied by a reduced relaxation response to the NO donor diethylamine (DEA)/NO and 8-Br-cGMP and associated expression of genes that mediate NO relaxation response (Reho & Fisher, 2015). Our lab reported that chronic blockade of NO in the presence of AngII had minimal change on blood pressure in MatSep rats compared to control rats that exhibited a dramatic increase in blood pressure. In contrast, MatSep rats showed increased heart rate compared to control rats (Loria, Kang, Pollock & Pollock, 2011). Total NOS and NOS isoform activity (NOS1, NOS2, and NOS3) were similar in MatSep rats compared to control rats as well as aortic NOS1 and NOS3 protein abundance. Because blood pressure regulation involves multiple organs and cell types, these findings suggest possible differences in NOS in the heart and in other organs. Additional studies are needed to determine NOS activity and NO production by immune cells after ELS and how it contributes to CVD in these animals.

Endothelin

Endothelin 1 (ET-1) is a potent vasoconstrictor peptide that exerts its action via binding to the endothelin A (ET_A) and endothelin B (ET_B) receptors. Both receptors are expressed on T cells, B cells, monocytes and neutrophils with pro-inflammatory effects when stimulated with ET-1 (Elisa et al., 2015). In addition, ET-1 increases the production of monocyte chemoattractant factor (MCP-1), ICAM-1 cell adhesion molecule, and macrophages (Saleh, 2010). Most of the pathological effects of ET-1 in CVD is mediated

through ET_A receptors. ET_B-deficient MatSep rats have elevated plasma ET-1 levels compared to wild type (WT) controls, with significantly lower levels of aortic ET_A and ET_B receptor expression evident in MatSep rats. Furthermore, in response to acute stress, control rats had elevated blood pressure response compared to MatSep rats, with similar levels of plasma ET-1 in both groups (Loria, Pollock & Pollock, 2010). Overall, available evidence indicates that early life adversity results in alteration of the ET-1 pathway in ET_B deficient MatSep rats, but more detailed study is needed to understand the effect of early life adversity on the immune ET system.

Sex differences

Sex difference adds another layer of complication to the effects of early life adversity on immune dysregulation. As in human studies, sex differences exist in rodent models used to explore the impact of early life adversity on inflammation and CVD. Studies from our laboratory demonstrated that chronic AngII infusion increases renal T cell numbers and renal damage score in male, but not in female, MatSep rats. Even though blood pressure is significantly increased in females MatSep rats, the increase is delayed until the second week of AngII infusion. In contrast, males have elevated blood pressure response from the first week of AngII infusion. Testosterone levels are higher and estrogen levels are lower in male MatSep rats compared to control rats (but not in females), and castration reduces blood pressure in males after MatSep. These findings point to the differential regulation of sex hormones in CVD and suggests that sex hormones contribute to differences in ELS-related hypertension (Loria, Yamamoto, Pollock & Pollock, 2013); however, the involvement of sex hormones in the altered immune cell function after ELS has not been investigated.

THE ROLE OF THE KIDNEYS IN HYPERTENSION AND INFLUENCES OF IMMUNE CELLS

Earlier work in kidney transplantation studies supported the importance of the kidneys in developing hypertension. Kidneys from hypertensive rats transplanted into normotensive rats caused hypertension in the normotensive rats. On the contrary, transplanted kidneys form normotensive rats caused a significant reduction in blood pressure in hypertensive rats, all pointing to the role of genetics and renal intrinsic functions in regulating blood pressure (Bianchi, Fox, Di Francesco, Giovanetti & Pagetti, 1974; Dahl & Heine, 1975; Dahl, Heine & Thompson, 1974; Kawabe, Watanbe, Kumiko & Sokabe, 1978). The same findings hold true in human kidney failure patients who were hypertensive. Over 4 years after receiving kidneys from normotensive individuals, those patients were reported to have normal blood pressure and a reversal in hypertension-induced heart and blood pressure damage (Curtis et al., 1983).

Genetic rodent models and pharmacological interventions have also supported the significant role of immune cells and hypertension. *Rag1* knockout mice first showed that genetic deletion of T cells, and not B cells, led to a significant decrease in AngII-hypertension (Guzik et al., 2007). Since then, several studies have been conducted to tease apart the role of other immune cells and cytokines in hypertension.

T cells

In severe combined immune deficiency (SCID) mice, which is characterized by lymphocyte deficiency, mean arterial pressure (MAP), systolic and diastolic blood pressure were blunted in AngII hypertension compared to mice with normal immune function (Crowley, Song, Lin, Griffiths, Kim & Ruiz, 2010). Further observation showed

significant reduction in glomerular damage, renal interstitial inflammation and a significant elevation in renal nitric oxide and prostaglandins thereby promoting natriuresis (Crowley, Song, Lin, Griffiths, Kim & Ruiz, 2010). In another study, Mattson et al used zinc finger nuclease technology to target Rag1 gene causing a frame-shift mutation in the Rag 1 gene and its deletion in the thymus in Dahl salt-sensitive rats (Mattson, Lund, Guo, Rudemiller, Geurts & Jacob, 2013). When fed a high salt diet, MAP and urinary albumin excretion were lower in the Rag1 mutants followed by a reduction in infiltrating immune cells into the kidneys (Mattson, Lund, Guo, Rudemiller, Geurts & Jacob, 2013). Furthermore, mice lacking Rag1 also showed blunted blood pressure and superoxide production in DOCA salt hypertensive model (Guzik et al., 2007). Furthermore, the role of the renal immune cells has also been studies in male and female animals. In Dahls salt sensitive rats fed a high fat diet, blood pressure, the proportion of $CD3^+$ and $CD4^+$ T cells, Th17 cells, and pro-inflammatory IL-17 cytokine in the aorta and kidneys was significantly increased similarly in males and females, while decreasing the anti-inflammatory Tregs cells compared to controls not fed a high fat diet (Taylor, Gillis, Musall, Baban & Sullivan, 2018). These findings point to the importance of T lymphocytes in promoting hypertension in males and females and in different models of hypertension.

Other studies began examining the involvement of T cell subsets in hypertension. Trott et al showed that genetic deletion of CD8 T cells, and not CD4 T cells, led to decrease in systolic and diastolic blood pressure and that adoptive CD8 T cells alone significantly raised blood pressure in AngII infused Rag1 knockout animals (Trott et al., 2014). CD8 T cells are also implicated in stimulation of the activity of distal tubular

sodium-chloride co-transporter leading to sodium retention and consequently hypertension in DOCA salt model (Liu et al., 2017). On the contrary, the adoptive transfer of CD4 T cells from placental from placental ischemic rats led to elevated blood pressure and oxidative stress in normal pregnant rats (Wallace et al., 2014). In pulmonary arterial hypertension (PAH), even though CD4 T cells were lower in PAH compared to normal patients with no changes in CD8 T cells, when stimulated in vitro, CD4 T cells produced significantly greater amounts of IL-17 suggesting the involvement of CD4 T cells in PAH through its polarization to Th17 cells (Hautefort et al., 2015).

Regulatory T cells (Tregs) serve a protective role in models of hypertension. By tail cuff and telemetry methods of blood pressure recording, adoptive transfer of Tregs in mice significantly lowered blood pressure in AngII treated mice including reduction in macrophage and T cell infiltration in the aorta (Barhoumi et al., 2011). The same group used a different animal model to study the role of Tregs in AngII hypertension. They injected T cells from wild type (WT) mice, or T cells from Scurfy mice, which is mice that is deficient in Tregs due to a mutation in the transcription factor forkhead box P3 (FOXP3) gene, or Tregs from WT mice, or T cells from Scurfy mice in combination with Tregs from WT mice (Mian, Barhoumi, Briet, Paradis & Schiffrin, 2016). Adoptive transfer of Tregs not only blunted systolic blood pressure in the vehicle AngII treated group, but it delayed systolic blood pressure elevation in the Scurfy group in response to AngII treatment (Mian, Barhoumi, Briet, Paradis & Schiffrin, 2016). Again, these findings support the protective role of Tregs and further supports the importance of T cells in hypertension.

Monocytes/Macrophages

Monocytes are a type of white blood cells that can differentiate into macrophages. Macrophages are antigen presenting cells and are part of the innate and adaptive immune system. Macrophage recruitment to sites of inflammation is controlled by monocyte chemoattractant protein-1 (MCP-1) through C-C chemokine receptor 2 (CCR2). CCR2 knock out animals had decreased aortic macrophages reduced vascular remodeling in AngII hypertension even though no changes in blood pressure was observed (Ishibashi et al., 2004). On the other hand, in AngII/high salt hypertensive model, CCR2 inhibition delayed blood pressure elevation, decreased renal monocyte and macrophage infiltration, lowered renal NFkB activity, TNF-α, and intracellular adhesion molecule 1 (ICAM1) (Elmarakby et al., 2007). These same findings are true in DOCA salt hypertensive model and in homozygous osteopetrotic (OP/OP) mice that has a deficiency in macrophage colony-stimulating factor (m-CSF) (Chan et al., 2012; De Ciuceis, Amiri, Brassard, Endemann, Touyz & Schiffrin, 2005). In those studies, blood pressure was reduced, and macrophages were significantly lowered in the aorta when CCR2 is inhibited (Chan et al., 2012), as well as reduced endothelial dysfunction, oxidative stress and reduced vascular remodeling in OP/OP mice treated with AngII (De Ciuceis, Amiri, Brassard, Endemann, Touyz & Schiffrin, 2005). Also, monocyte and macrophage depletion using liposome-encapsulated clodronate (LEC) in AngII hypertensive model led to lower blood pressure, reduced renal macrophage infiltration, albuminuria, renal pro-inflammatory TNF-α and IL-1β, and oxidative stress (Huang et al., 2018). In Dahl salt sensitive rats, LEC treatment also lowered salt-induced blood pressure elevation and circulating monocytes (Huang et

al., 2018). Overall, these studies show that in several models of hypertension, macrophages are important therapeutic target for treating hypertension.

Dendritic cells

Dendritic cells (DCs) present antigens on its cell surface for T cell recognition and activation during immune response. During hypertension, modified proteins also known as isoketals generated and accumulate in DCs. These accumulation of isoketals in DCs leads to cytokine production by the activated DCs and co-stimulatory molecules like CD80 and CD86 thereby promoting T cell proliferation and production of pro-inflammatory cytokines such as IFN-γ and IL-17 as well as hypertension in AngII and DOCA salt hypertensive models. Adoptive transfer of DCs from AngII treated mice induced T cell proliferation and pro-inflammatory cytokine production; whereas, isoketal scavenger, attenuated the DCs response and hypertension (Kirabo et al., 2014). Another study, although not showing any direct evidence for the role of DC in hypertension, did reveal by intravital microscopy in a reporter mouse that renal DCs led to T cell migration into the kidneys, an indication that DCs may play an important role in renal inflammation and possibly hypertension (Yatim, Gosto, Humar, Williams & Oberbarnscheidt, 2016). Unfortunately, studies have not been done to genetically deplete dendritic cells in order to determine its direct role in hypertension, renal function, and vascular dysfunction.

Natural killer cells

Natural killer cells are part of the innate immune system and is implicated in hypertension. During hypertension immune cells are recruited to the kidneys by intravasation through the blood vessels, a crucial part of hypertension induced vascular and renal damage. In AngII hypertension, IFN-γ mediates inflammation. IFN-γ

deficiency model showed that vascular dysfunction and inflammation is dependent on IFN-γ in AngII hypertension (Kossmann et al., 2013). Interestingly, the recruitment of natural killer cell receptors was significantly reduced in the aortic wall in the absence of IFN-γ. The authors further utilized selective depletion and adoptive transfer techniques to show that observed vascular inflammation was dependent on the activation of monocytes and natural killer cells in AngII hypertension (Kossmann et al., 2013). Also, the role of natural killer cells in hypertension induced-vascular inflammation is strain dependent as shown by Taherzadeh et al (Taherzadeh et al., 2010). The BALB.B6-Cmv1r expressed the natural killer cell gene complex of C57BL/6 strain and showed similar sensitivity to developing LNAME-induced hypertension and vascular dysfunction as the C57BL/6 mice compared to the control BALB/c background (Taherzadeh et al., 2010). These findings are indications that natural killer cells play an important role in hypertension and in hypertension-induced vascular dysfunction.

Cytokines

Cytokines produced by immune cells are also implicated in hypertension. In AngII model of hypertension, IL-17 is significantly elevated, but genetic deletion of IL-17 gene showed significant reduction in both systolic and diastolic blood pressures as well as reduction in superoxide production in the vessels (Madhur et al., 2010). Similarly, in preeclampsia and in DOCA- salt hypertensive model, IL-17 receptor blockade blunted blood pressure (Amador et al., 2014; Cornelius et al., 2013). IFN-γ is also produced by T cells, more so by memory T cells. T cell memory was induced in an IFN-γ knockout mice by first treating the animals with L-NAME to induce hypertension and to stimulate IFN-γ production by effector T cells (Itani et al., 2016). After a washout period, the effector T

cells die but some of the effector T cells become long-lived memory T cells. The same animals were then placed on a high salt diet to stimulate the production of IFN-γ by those long-live effector memory T cells (Itani et al., 2016). Compared to wild-type animals, IFN-γ knockout lead significant reduction in AngII induced blood pressure elevation on a high salt diet (Itani et al., 2016). Another study shows that IFN-γ production also contributes to blood pressure elevation in the absence of memory T cells (Kamat et al., 2015). On the contrary, IFN-γR or T-bet (a transcription factor involved in IFN-γ production in T cells) knockout animals did not show any changes in AngII induced hypertension (Marko et al., 2012; Zhang et al., 2014). These findings suggest that IFN-γ produced by effector T cells and long-lived memory T cells influence blood pressure.

In a model of autoimmunity, NZBNZWF1 mice developed hypertension between age 6 to 12 weeks old compared to the control normotensive, NZW, animals (van Heuven-Nolsen et al., 1999). The authors used neutralizing antibodies against IL-4 and IFN-γ to determine the involvement of humoral and cell mediated immunity respectively in order to understand their involvement in blood pressure regulation in an autoimmune disease model. The study revealed that treatment with neutralizing antibody against IL-4 significantly lowered blood pressure in NZBNZWF1 compared to the control NZW mice. However, the opposite effect was observed upon treatment with neutralizing antibody against IFN-γ, thus suggesting that IL-4 is the contributing cytokine to elevated blood pressure in an autoimmune disease model (van Heuven-Nolsen et al., 1999).

AngII models of hypertension have also shown elevated plasma levels of IL-6 (Lee et al., 2006). Although blood pressure was similar in IL-6 knock out and wild type control mice when placed on a high salt diet, chronic AngII infusion in addition to

continuous high salt diet led to a significantly lower blood pressure in IL-6 knock out animals compared to wild type controls, lower urinary albumin excretion (Lee et al., 2006) and protects against endothelial dysfunction (Schrader, Kinzenbaw, Johnson, Faraci & Didion, 2007). Conversely, the significant decrease in blood pressure in IL-6 knock out animals during AngII infusion did not hold true in the DOCA salt hypertensive models (Sturgis, Cannon, Schreihofer & Brands, 2009). No blood pressure differences were observed between IL-6 knock out and wild type controls when placed on DOCA salt treatment (Sturgis, Cannon, Schreihofer & Brands, 2009).

Cytokines produced by macrophages also contribute to hypertension. IL-1 is a highly pro-inflammatory cytokine expressed in almost every cell. It exerts its function by binding to the IL-1 receptor. In a study conducted by Zhang et al, they utilized an IL-1 receptor knockout mice to show that IL-1R deficiency inhibits blood pressure elevation and promotes natriuresis by promoting nitric oxide production in intrarenal macrophages thereby inhibiting sodium-potassium-two chloride transporter activity in the kidneys (Zhang et al., 2016). Furthermore, in TNF-α knockout animals, blood pressure and cardiac hypertrophy was significantly attenuated in AngII-induced hypertensive model compared to wild type animals. TNF-α treatment restored AngII-induced blood pressure elevation and cardiac hypertrophy in knockout animals (Sriramula, Haque, Majid & Francis, 2008). Zhang et al used renal transplantation model to show that wild type animals who received kidneys from TNF-α knockout animals exhibited lower blood pressure and higher nitric oxide production (Zhang et al., 2014).

The role of cytokines produced by regulatory T cells have also been studied in models of hypertension. IL-10 is produced by Tregs among other cells and has anti-

inflammatory properties. During preeclampsia, the hypertension induced by DOCA salt treatment was significantly attenuated when recombinant IL-10 was given to pregnant rats followed by improved endothelial and renal function, reduced plasma ET1 and reduced platelet endothelial cell adhesion molecule 1 (PECAM-1) in the aorta and placenta (Tinsley, South, Chiasson & Mitchell, 2010). IL-10 deficiency resulted in endothelial dysfunction which was reversed by treatment with superoxide scavengers, PEG superoxide dismutase and tempol in AngII hypertension (Didion, Kinzenbaw, Schrader, Chu & Faraci, 2009; Lima et al., 2016). The influence of IL-10 on hypertension is also through increasing the production of chemokine CCL5 in vascular smooth muscle cells via angiotensin II receptor (AT2R) activation in spontaneously hypertensive (SHR) rats treated with AngII (Kim & H.S., 2014), and by increasing Tregs which led to significant decreases in cytokines such as IL-6 and TNF-α in preeclampsia (Harmon et al., 2015).

Transforming growth factor beta (TGF-β) is another cytokine produced primarily by Tregs. It prevents the production of activation and proliferation of T cells, macrophages and B cells (Rodriguez-Iturbe, Pons & Johnson, 2017). TGF-β is stimulated on a high salt diet and as such may play a role in salt sensitive hypertension. Indeed, treatment with anti TGF-β antibody not only significantly decreased blood pressure in Dahl salt sensitive rats but also decreased proteinuria, glomerulosclerosis, and renal fibrosis an indication of the protective role of TGF-β in salt-induced hypertension (Dahly, Hoagland, Flasch, Jha, Ledbetter & Roman, 2002). These renal protection was also observed in both male and female Dahl salt sensitive rats (Murphy et al., 2012).

IL-2 is produced by activated T cells (Rodriguez-Iturbe, Pons & Johnson, 2017). IL-2 and Tregs depend on each other for survival. IL-2 is important for the generation of Tregs and its survival and IL-10 production, in return, Tregs regulates IL-2 availability by utilizing it for inhibiting costimulation of CD80/86 (de la Rosa, Rutz, Dorninger & Scheffold, 2004; Rodriguez-Iturbe, Pons & Johnson, 2017). IL-2 administration decreased blood pressure (Ishimitsu, Uehara, Numabe, Tsukada, Ogawa & Yagi, 1993; Tuttle & Boppana, 1990), creatinine clearance, and reduced glomerular and arterial injury in Dahl salt-sensitive rats (Ishimitsu, Uehara, Numabe, Tsukada, Ogawa & Yagi, 1993). To sum it all, pro-inflammatory cytokines promotes hypertension, renal and endothelial dysfunction while anti-inflammatory cytokines attenuates hypertension and hypertension induced renal injury in various animal models of hypertension.

Pharmacological intervention

The use of pharmacologic intervention to inhibit immune cells have also proven to be beneficial in hypertension. Mycophenolate mofetil is an immunosuppressive agent that have been used in high fat diet (HFD)-induced hypertension in Dahl salt sensitive rats to show a reduced blood pressure, lower numbers of T cells in the glomeruli, reduced adiposity, and improvement in glomerular injury compared to control rats an indication that immune cells contribute to HFD-induced hypertension (Spradley, De Miguel, Hobbs, Pollock & Pollock, 2013). In the same Dahl salt sensitive rats, suppressing immune cells by treating with MMF also decreased blood pressure, lower T cell infiltration and cytokine concentration in the kidneys, and improved kidney function, and increased nitric oxide bioavailability in high protein diet, high salt diet, and DOCA salt-induced

hypertensive rats (De Miguel, Das, Lund & Mattson, 2010; De Miguel, Lund & Mattson, 2011; Moes et al., 2018).

Etanercept is another immunosuppressant that blocks the pro-inflammatory effects of TNF-α and influencing blood pressure in hypertensive models. Renal interstitial infusion of etanercept significantly reduced blood pressure elevation, glomerular injury and fibrosis in Dahl salt sensitive rats (Huang et al., 2016). In DOCA salt hypertension model, even though etanercept did not lower blood pressure, it did significantly reduce proteinuria, MCP-1 and ET-1 urinary excretion, and cortical NF-kB activity (Elmarakby, Quigley, Imig, Pollock & Pollock, 2008). AngII/high salt treated rats showed lower MCP-1 excretion and reduced macrophage positive staining in renal cortex and these findings were attributed to the increase in Cyp2c23 enzymes in renal vasculature, an indication of improved endothelial function due to TNF-α inhibition (Elmarakby, Quigley, Pollock & Imig, 2006). In renal failure, animals treated with TNF-α neutralizing molecule, PEG-sTNFR1, showed reduced blood pressure, renal inflammation, albuminuria, NF-kB activity, ET-1 production, and increase in NO production (Therrien, Agharazil, Lebel & Lariviere, 2012). Additionally, etanercept treatment improved vascular function, prevented hypertension, and restored NO synthase expression in the thoracic aorta of fructose-induced hypertensive rats (Tran, MacLeod & MbNeill, 2009).

CHAPTER 3

MATERIALS AND METHODS

Animals

All animal protocols were approved by the Institutional Animal Care and Use Committee at Augusta University and the University of Alabama at Birmingham. Wistar Kyoto (WKY) rat breeding pairs were purchased from Charles River (Hartford, CT) and acclimatized to the animal housing facility for 2 weeks. Rats were fed a standard diet (Teklad 8604, Madison, WI) with free access to water and food. All animals used for the experimental procedures were housed in a controlled 12:12 hr light:dark cycle.

Maternal separation protocol

The maternal separation (MatSep) protocol was performed using offspring from WKY breeders, as previously described (Loria, Pollock & Pollock, 2010). Briefly, from postnatal day 2 to 14, male pups were removed from the dam and placed in a 30°C incubator with clean bedding for 3 hr/day with constant humidity. MatSep male rats were identified by having their tails snipped and cauterized with silver nitrate. Non-separated male littermates were used as control rats. Weaning occurred on postnatal day 28 and experiments were carried out in adulthood at 12 weeks of age. Control and MatSep rats were randomly selected from different litters and assigned to their respective experimental groups.

Blood, kidney, kidney vessels, and spleen preparation

Blood.

Blood collected from the abdominal aorta was gently overlaid on 5 ml of the density separating agent Histopaque (Sigma-Aldrich), centrifuged at 400 x *g* for 30 min at room temperature. The buffy coat containing mononuclear cells was resuspended, washed 3 times in phosphate buffered saline (PBS) and numbers of mononuclear cells were determined under the microscope using a hemocytometer. Cells were then used in the flow cytometry or magnetic bead separation protocols described below.

Kidney. Kidneys were harvested, flash-frozen in liquid nitrogen and maintained at -80^{o}C for ELISA or quantitative real time-polymerase chain reaction (qRT-PCR) analysis. For isolation of mononuclear cells and T lymphocytes, a subset of kidneys were cut in 1- to 2-mm-thick sections and incubated in digestion solution for 1 hour at 37^{o}C as previously described (De Miguel, Das, Lund & Mattson, 2010) and mononuclear cells isolated by density separation over histopaque. T lymphocytes were then isolated from the mononuclear fraction by following the magnetic bead separation protocol below. The number of isolated mononuclear cells and T cells were counted using a hemacytometer to determine the absolute number of cells, and isolated T cells were also processed for qRT-PCR as described below.

Kidney vessel isolation.

A subset of harvested kidneys was quickly placed in ice-cold Hanks balanced salt solution containing phenylmethyl sulfonyl fluoride (PMSF, 2 mM) and protease inhibitors (10 μM leupeptin, 2 μM pepstatin, and 0.0001% aprotinin). After removal of

the renal capsule, a 70 μm sieve mesh (BioDesign, Carmel, NY) was wrapped around the kidney. Renal vessels were isolated by gently grating the tissue with a spatula until only the kidney vessel tree was visualized (Schneider, Wach, Durley, Pollock & Pollock, 2010). Kidney vessels were then flash frozen in liquid nitrogen and kept at -80˚C for analysis of gene expression by qRT-PCR as described below.

Spleen and Aorta. Spleens and thoracic aortae were quickly harvested, flash frozen in liquid nitrogen and kept at -80ºC for analysis of cytokine levels by ELISA as described below.

Flow cytometry

Circulating mononuclear cells were resuspended in 100 μl of 0.5% BSA PBS and incubated on ice for 15 minutes in the presence of allophycocyanine-conjugated anti-rat CD3 (clone IF4), fluorescein isothiocyanate-conjugated anti-rat CD4 (clone OX-35) and CD11b (clone OX-42), phycoerythrin-conjugated anti-CD45R (clone HIS24) and CD8 (clone OX-8), peridinin chlorophyll-conjugated anti-rat CD8 (clone OX-8), and Alexa 647-conjugated anti-rat CD11C (clone 8A2) and Foxp3 (clone FJK-16s). All antibodies were purchased from BD Biosciences, San Jose, CA; eBioscience, San Diego, CA; and Serotec (Bio-Rad, Raleigh, NC). Data acquisition was performed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA) and analyzed using BD CellQuest™ software (BD Biosciences).

Isolation of T cells using magnetic beads

Circulating and kidney mononuclear cells were centrifuged at 300 x *g* for 10 min at room temperature, resuspended in staining buffer and incubated with MACS pan T-cell antibody coupled to magnetic microbeads for 15 minutes at 4ºC (Miltenyi Biotec, San

Diego, CA). Cells were then washed, centrifuged, and resuspended in 500 µl of staining buffer and applied to MACS magnetic separation columns (Miltenyi Biotec) to isolate T lymphocytes. Absolute numbers of isolated T lymphocytes were determined using a hematocytometer. Inflammatory gene expression was studied in isolated T cells by qRT-PCR as described below.

Quantitative real-time PCR

RNA isolation and cDNA conversion were performed using Qiagen (Valencia, CA) kits, following manufacturer's instructions. RNA concentration was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The QuantiTect Reverse Transcription Kit was used to convert 1 µg of RNA to cDNA by following the reverse transcription 2-step protocol. In step one, genomic DNA elimination reaction mix was added to the RNA and the reaction was incubated at 42°C for 5 min. The reaction was stopped by immediately placing the samples on ice. In *step 2*, the reverse transcription components were added to the reaction tube and incubated at 42°C for 15 min to activate the reverse transcriptase, then at 95°C for 3 min to inactivate the reverse transcriptase. Primers for rat IL-1β, TNF-α, IFN-γ complement component 3 (C3), chemokine (C-X-C motif) ligand 6 (CXCL6), CXCL11, CXCL2, CXCL9, chemokine (C-C motif) ligand 12 (CCL12), CCL19, CXCL1, and CCL3, from Qiagen were used for the amplification step (Catalog numbers: QT00187158 (C3), QT02465120 (CXCL6), QT00372302 (CXCL11), QT00184891 (CXCL2), QT00183316 (CXCL9), QT01604624 (CCL12), QT01592724 (CCL19), QT00185528 (CXCL1), and QT00378350 (CCL3)), using 1.25 µl of sample cDNA. 40 cycles of DNA amplification were performed (95°C for 15 sec and 55°C for 40 sec after the initial DNA denaturing

step (Bio-Rad CFX96™ Thermal Cycler. Hercules, CA). mRNA expression of circulating IL-1β, TNF- α and IFN-γ was normalized to GAPDH, and mRNA expression of C3, CXCL6, CXCL11, CXCL2, CXCL9, CCL12, CCL19, CXCL1, and CCL3 in whole kidneys of untreated and LPS treated MatSep rats were normalized to β -actin. Gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to expression levels in control animals, as described in the *Statistical Analysis* section.

Cytokine ELISA

Whole kidneys, spleen, and aorta from control and MatSep rats were homogenized in buffer containing 5.8mM PMSF, 2.3 μM leupeptin and 7.3 μM pepstatin and 0.5% Triton X-100. The homogenates were sonicated 3 times on ice and incubated at 4°C for 1 hr. After centrifugation at 20,000 x *g* for 20 min at 4°C, protein concentration in the supernatant extract was determined using Quick Start™ Bradford 1× Dye Reagent (BioRad, Hercules, CA). IL-1β, IL-4, IL-6, IFN-γ, and TNF-α protein levels in the supernatant extract were measured using rat-specific ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Levels of IL-17 and IL-10 were also analyzed in whole kidney homogenates (R&D Systems). All cytokine measurements were normalized to total protein. The minimum detectable level for IL-1β, IL-4 and TNF-α was 5 pg/mL, IFN-γ and IL-10 was 10 pg/mL, IL-17 was 15.6 pg/ml, and IL-6 ranges from 14-36 pg/mL.

Immunohistochemical staining and analysis

Control and MatSep kidneys were fixed in 10% neutral buffered formalin solution and embedded in paraffin. Kidney sections (0.4 μm) were deparaffinized in a 61°C incubator for 30 min. Tissues were then re-hydrated by placing the slides in several

changes of ethanol (2 × 100%, 2 × 95%, and 1 × 70%) and finally water. Exogenous peroxidase was blocked by incubating slides in 30% H_2O_2 for 20 min. Antigen retrieval was performed by placing the slides in a 1:10 dilution of target retrieval solution (DAKO, Carpinteria, CA) and steamed for 35 min. Non-specific binding was blocked using rodent block R (BioCare Medical, Concord, CA) for 20 min. Slides were incubated for 24 hr at 4°C in primary antibodies against IL-1β (1:2000), Toll-like receptor 4 (TLR4) (1:400; a receptor for LPS), Ki-67 (1:5,000; cell proliferation marker), myeloperoxidase (MPO) (1:2,000; activated neutrophil marker), cluster of differentiation 69 (CD69) (1:10,000; T cell activation marker) or CD44 (1:30,000, hyaluronan receptor, immune cell trafficking, leukocyte adhesion and activation marker) (Abcam, Cambridge, MA). DAB substrate (DAKO) was used to visualize positive stains for IL-1β, TLR4, Ki-67$^+$, MPO$^+$, CD69$^+$, and CD44$^+$.

Numbers of TLR4$^+$, Ki-67$^+$, and MPO$^+$ cells were quantified in renal cortex, outer and inner medulla by counting 10 random microscopic fields (400 □m x 400 □m, 200X magnification) per kidney region in a blinded manner. Whole kidney sections were analyzed for quantification of CD69$^+$ cells. For CD44$^+$ cell quantification, 60 random glomeruli/kidney were assessed, and the number of glomeruli presenting CD44$^+$ cells was quantified. Additionally, the number of CD44$^+$ cells/glomerulus was determined at 400X magnification. Data are expressed as number of positive cells/field. Immunostaining for TLR4 and CD44 was also assessed by analyzing whole kidney scans (100X magnification) captured with a scanning microscope fitted with a DP73 camera (Olympus America, Melville, NY). Cortical, outer and inner medullary areas of each kidney image were outlined using Metamorph imaging software (Molecular Devices, Sunnyvale, CA),

and the amount of positive staining for TLR4 and CD44, within those areas was quantified. Data are expressed as percentage of area of the kidney (cortex, outer or inner medulla) positively staining for TLR4 or CD44.

LPS treatment

Control and MatSep rats were given a single low dose of LPS (2 mg/kg, i.v.; Sigma-Aldrich, St. Louis, MO) or an equal volume of 0.9% NaCl (vehicle). Fourteen hours after LPS or vehicle treatment, animals were euthanized by a single injection of pentobarbital sodium (65 mg/kg). Blood and kidneys were collected for determination of absolute numbers of immune cells, inflammatory gene expression, and inflammatory cytokine levels. Spleens were collected for determinations of inflammatory gene expression and cytokine levels.

mRNA isolation and RT^2 profiler PCR array

Renal vessel and whole kidney mRNA isolation and cDNA preparation

In separate homogenization tubes containing lysis buffer, mRNA was isolated from renal vessels or whole kidneys using RNeasy Mini Kit (Qiagen) following manufacturer's instruction. Total RNA concentration was determined using a NanoDrop 2000 spectrophotometer. For reverse transcription PCR steps, 0.5µg of RNA was reverse transcribed using RT^2 First Strand Kit (Qiagen) following manufacturers instruction.

PCR arrays: Qiagen RT^2 Profiler PCR Arrays were used to determine differential gene expression of 84 genes in circulating T-cells and whole kidney (vehicle and LPS treated) (Rat Inflammatory Cytokines and Receptors; PARN-011A) as well as in renal vessels (Rat T_H17 Autoimmunity and Inflammation; PARN-073A) of control and MatSep rats. Gene analyses were performed using a 2 step PCR protocol with 40 cycles of 95°C for 15

sec and 55°C for 40 sec after the initial DNA denaturing step (Bio-Rad CFX96™ Thermal Cycler. Hercules, CA). The cycle threshold (Ct) values for individual genes were subtracted from the average of 5 housekeeping genes included in PCR array plate, reported as change in Ct (ΔCt). Each control and MatSep values were normalized to the appropriate control group by subtracting each ΔCt values from the average ΔCt values of the control group to yield ΔΔCt. The reported values were represented as fold change calculated as as $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data are presented as mean ± S.E.M. Unpaired Student's t test was used to determine differences between control and MatSep rats for cytokine mRNA and protein expression, immunostaining for Ki-67, CD69, CD44, MPO and TLR4 and absolute numbers of circulating and kidney mononuclear cells and T-lymphocytes. Differences in circulating immune cell populations detected by flow cytometry were evaluated using one-way ANOVA. Differences in cytokine expression levels assessed by PCR arrays were analyzed using the nonparametric Mann-Whitney test. Significant differences were defined as $P < 0.05$ using GraphPad Prism Software (San Diego, CA).

AIM 2

Animals

Animals are the same as described in AIM 1

Maternal separation protocol

Maternal separation protocol is the same as described in AIM 1

Telemetry implantation and blood pressure recording

Under anesthesia with 2-3% isoflurane, the abdominal aorta of 11 weeks old control and MatSep male rats was exposed by making a midline incision for telemetry transmitter (Data Sciences International, Duluth, MN) implantation, while briefly occluding the aorta to prevent blood loss. An incision was made distal to the occluded part of the aorta and the transmitter catheter was inserted and glued (Vetbond, 3M Corporation, St. Paul, MN) to ensure stability. The transmitter body was held in place by suturing it to the abdominal wall as the incision is being closed. Wound staples were used to bind the skin. Rats were then housed in individual cages in a telemetry designated room and left undisturbed to recover for 7 days after surgery before beginning LNAME infusion. Telemetry receivers were placed on the top of every cage and mean arterial pressure (MAP) and heart rate (HR) was recorded at 10-second intervals every 10 minutes for the duration of the study.

LNAME treatment

Baseline blood pressure was recorded for four days after recovery from telemetry surgery. At the end of day 4, LNAME (Sigma, St. Louis, MO, USA), 100mg/kg/day, was prepared daily and given to experimental animals in drinking water for 7 days. After day 7, animals were killed, and blood pressure recording was analyzed using Excel software.

Metabolic cages

In a separate group, rats were treated with LNAME while in their home cage. On day 5, individual rats were placed in metabolic cages and allowed to acclimate for a day. On day 7, 24 hours urine was collected and frozen at minus 80-degree Celsius for

analyses of proteinuria, glomerular, and tubular damage. Additionally, the volume of LNAME consumed was measured for normalization of urinary measurements.

Proteinuria, urinary glomerular and tubular damage marker measurements

Proteinuria was measured using Bradford assay (Bio-Rad). Urinary glomerular damage markers, albuminuria (GenWay, San Diego, CA, USA) and nephrin (myBioSource, San Diego, CA, USA), and urinary tubular damage markers, KIM-1 (R&D Systems, Minneapolis, MN, USA) and NGAL (Abcam, Cambridge, MA, USA) was determined by ELISA. Urinary excretion rates were calculated using 24 hours urine volume collected from metabolic cages as described above.

Flow cytometry

On Day 7 of LNAME treatment, kidneys were flushed with 20mL ice-cold saline through the left ventricle of the heart. Immune cells were isolated from whole kidneys, minced, and digested in collagenase (Sigma, St. Louis, MO, USA) in 5mL DMEM high glucose (Thermo Scientific, Waltham, MA, USA) in the absence of fetal bovine serum, in warm bath while shaking at 37^0C for 30 minutes. After digestion, 10mL of stop solution containing 1X PBS, 0.5% bovine serum albumin and 2mM EDTA. Kidney tissue was further broken apart by passing the tissues through a 20-gauge needle and filtered through 70μm filter before passing it further through a 40μm filter. The homogenate was then centrifuged at 1670rpm, for 10 minutes at 4^0C. Red blood cells were lysed with 50mL ACK lysis buffer for 3 minutes at room temperature. The reaction was stopped by adding 50mL of 1X PBS, spun at 1670rpm for 10minutes at 4^0C. The resulting pellet was resuspended in 1mL PBS and the number of cells counted using a hemocytometer. Cells were incubated in fixable viability dye to discriminate between viable and non-viable

cells. After washing and resuspending the pellet in staining buffer, non-antigen specific binding of immunoglobulins to Fcγ receptors was blocked by adding purified rat anti-mouse CD16/CD32. Cells were then incubated in 5μg/ml mouse anti rat antibodies as follows: FITC conjugated anti-CD3 (clone G4.18), phycoerythrin conjugated anti CD45 (clone OX-1), peridinin chlorophyll protein complex conjugated anti CD8 (clone OX-8), and allophycocyanin cyanine 7 conjugated CD4 (OX35) on ice for 30 minutes. All antibodies used were purchased from BD Biosciences (San Jose, CA, USA). All data was acquired on the LSRII flow cytometer (BD Biosciences, San Jose, CA, USA) utilizing FACSDiva software (BD Biosciences, San Jose, CA, USA). All data acquired were analyzed using FlowJo software (Tree Star Inc, Ashland, OR, USA).

Statistical analysis

All data are presented as mean ± S.E.M. Student's t-test was used to determine statistical significance for blood pressure measurements. Renal damage markers and flow cytometry statistical analyses was determined by 2 way-ANOVA with a Tukey's post hoc analyses. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA), and statistical significance set at $P < 0.05$.

CHAPTER 4

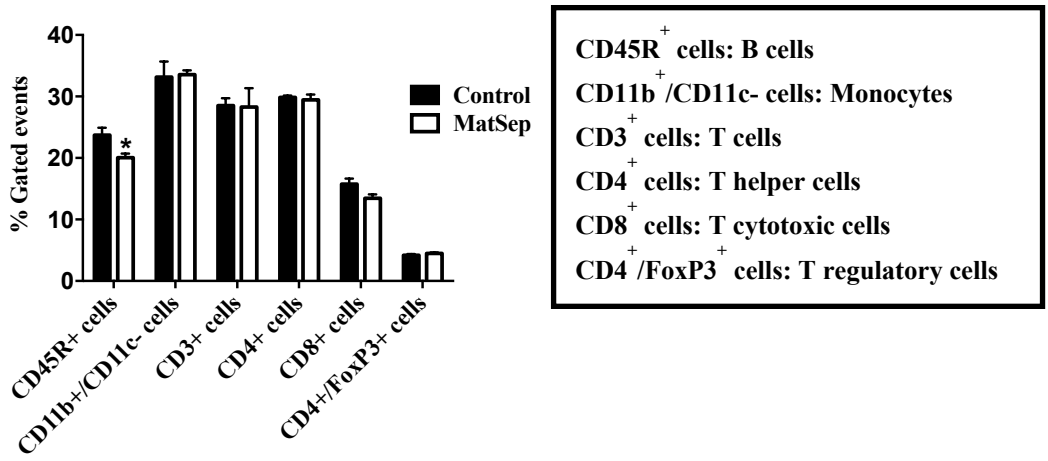
RESULTS

AIM 1: To characterize the renal inflammatory state in adult male rats

MatSep does not change numbers of macrophages or T lymphocytes in adult male rats

The data obtained by flow cytometry revealed that the percentage of gated events of circulating macrophages, total T cells, helper, cytotoxic, and regulatory T cells ($CD11b^+/CD11c^-$, $CD3^+$, $CD4^+$, $CD8^+$, and $CD4^+/FoxP3^+$ cells respectively) were not altered by MatSep (Figure 3A). Paradoxically, circulating percentages of B cells ($CD45R^+$ cells) were significantly decreased in MatSep rats compared to control rats (Figure 3A). Similarly, absolute numbers of circulating and kidney mononuclear and T cells did not differ between MatSep and control rats (Figure 3B).

(A)



(B)

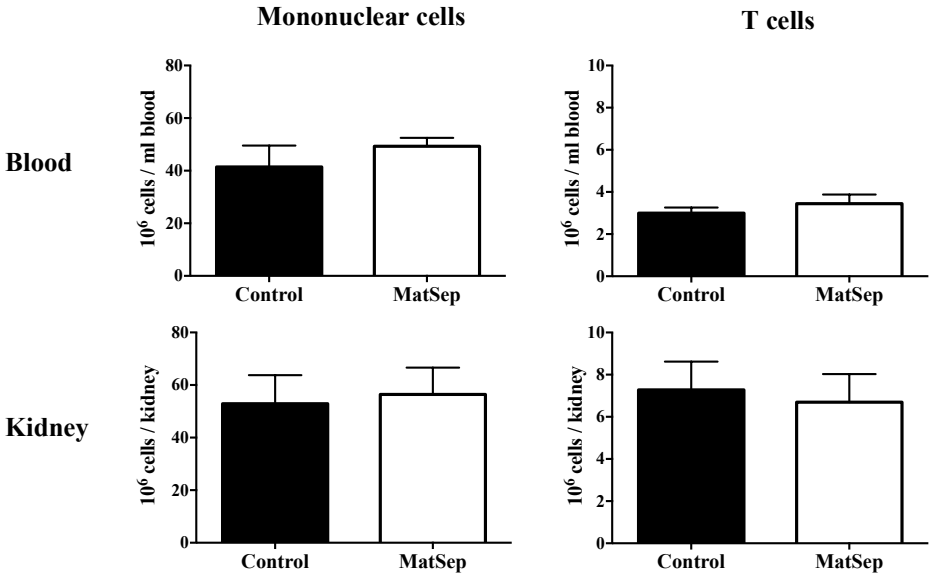


Figure 3. (A) Percent gated events of circulating immune cells; (B) Absolute numbers of mononuclear and T cells in blood and kidney of control and MatSep rats. Results are expressed as mean ± s.e.m. *$P < 0.05$ vs. control. n=4-6/group.

MatSep induces increased renal levels of IL-1 β and TLR4 in adults

Inflammatory gene PCR array analysis of 84 cytokine and cytokine receptor genes in isolated circulating T-cells and renal vessels revealed no differences in gene expression in MatSep rats when compared to control rats. Specific analysis with RT-PCR of mRNA expression of IL-1β, TNF-α, and IFN- γ in isolated circulating T cells revealed significantly increased expression of IL-1β in MatSep rats compared to control rats [fold change vs. control, (95% confidence interval); 3.41, (0.58, 4.25), p = 0.0130], whereas mRNA expression of TNF-α [0.807, (-0.38, 0.76), p=0.476] and IFN- γ [1.05, (-0.92, 0.81), p=0.896] was similar in MatSep and control rats.

ELISAs were performed to determine cytokine levels in kidneys from control and MatSep rats. Interestingly, MatSep led to increased renal levels of IL-1β when compared to control (Table 1), while no differences in renal IL-4 and IL-6 were observed (Table 1). Renal levels of IFN- γ, IL-17 and IL-10 were below the level of detection (<10 pg/ml for IFN- γ and IL-10, <15.6 pg/ml for IL-17) in both control and MatSep groups.

Table 1. Protein levels of IL-1β, IL-4, IL-6, TNF-α, and IFN-γ in whole kidneys and spleens of control and MatSep rats.

Cytokine	Control (pg/mg protein ± SEM)	MatSep (pg/mg protein ± SEM)	P value
KIDNEY			
IL-1β	4.44 ± 0.476	7.91 ± 1.02	0.0186*
IL-4	4.52 ± 1.04	3.98 ± 1.87	0.788
IL-6	6.71 ± 0.647	5.96 ± 1.10	0.578
TNF-α	ND	ND	
IFN-γ	BD	BD	
SPLEEN			
IL-1β	146.1 ± 11.15	145.4 ± 9.34	0.96
IL-4	0.74 ± 0.048	0.50 ± 0.080	0.024*
IL-6	3.22 ± 0.15	2.90 ± 0.27	0.32
TNF-α	0.94 ± 0.048	1.02 ± 0.068	0.32
IFN-γ	9.21 ± 0.48	7.61 ± 0.33	0.016*

*$P < 0.05$ vs. control. n=4-9/group. BD = below detection, ND = not detected

Immunohistochemical staining was used to localize expression of IL-1β in the kidney. The intensity of IL-1β staining was higher in all regions of MatSep compared to control rats (Figure 4). Particularly, higher intensity of IL-1β was distinct in the distal nephron tubular epithelial cells of MatSep rats compared to control rats (Figure 4).

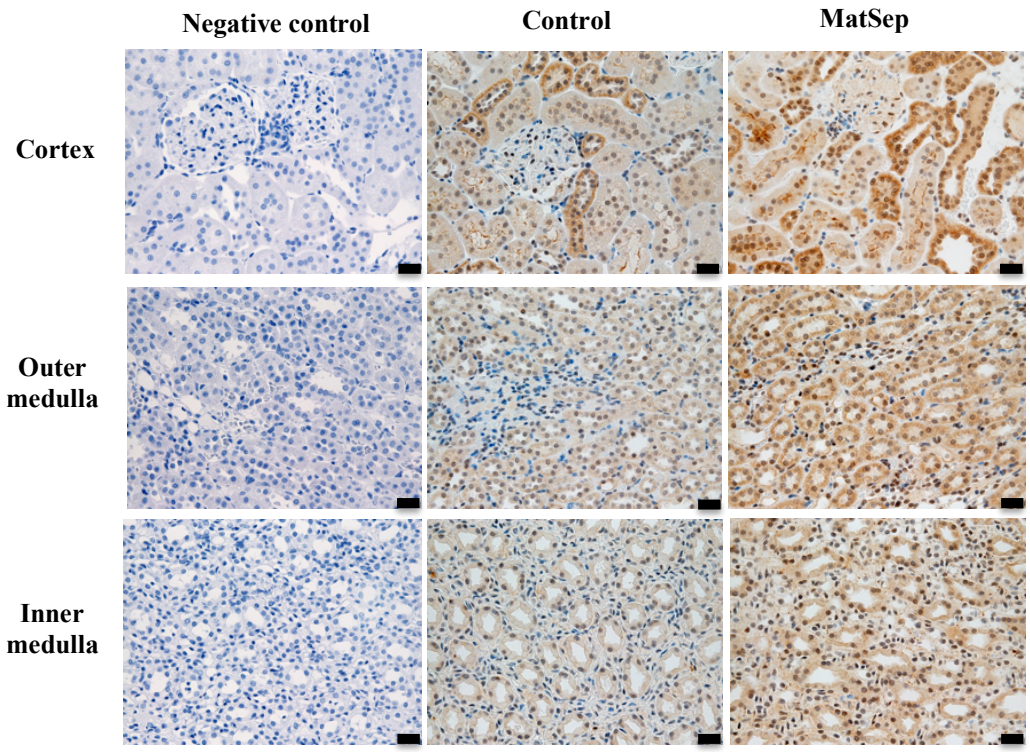


Figure 4. Representative images of IL-1β protein expression in the renal cortex, outer medulla, and inner medulla of control and MatSep rats. n=6-8/group. Scale bar = 20μm.

Toll-like receptors (TLR), especially TLR4, have been linked to the expression of pro-inflammatory cytokines such as IL-1β (Grishman, White & Savani, 2012; Medzhitov, 2001). Thus, we further probed the expression of TLR4 in the kidney sections. Interestingly, TLR4 expression was prominent in the brush border of proximal tubules in both control and MatSep rats similarly (Figure 5A). TLR4 expression in the renal medulla was observed in renal interstitial cells (Figure 5A). Analysis of the number of TLR4 immunopositive cells in the renal medulla showed that MatSep rats had

significantly increased numbers of TLR4 positive cells compared to control rats (Figure 5).

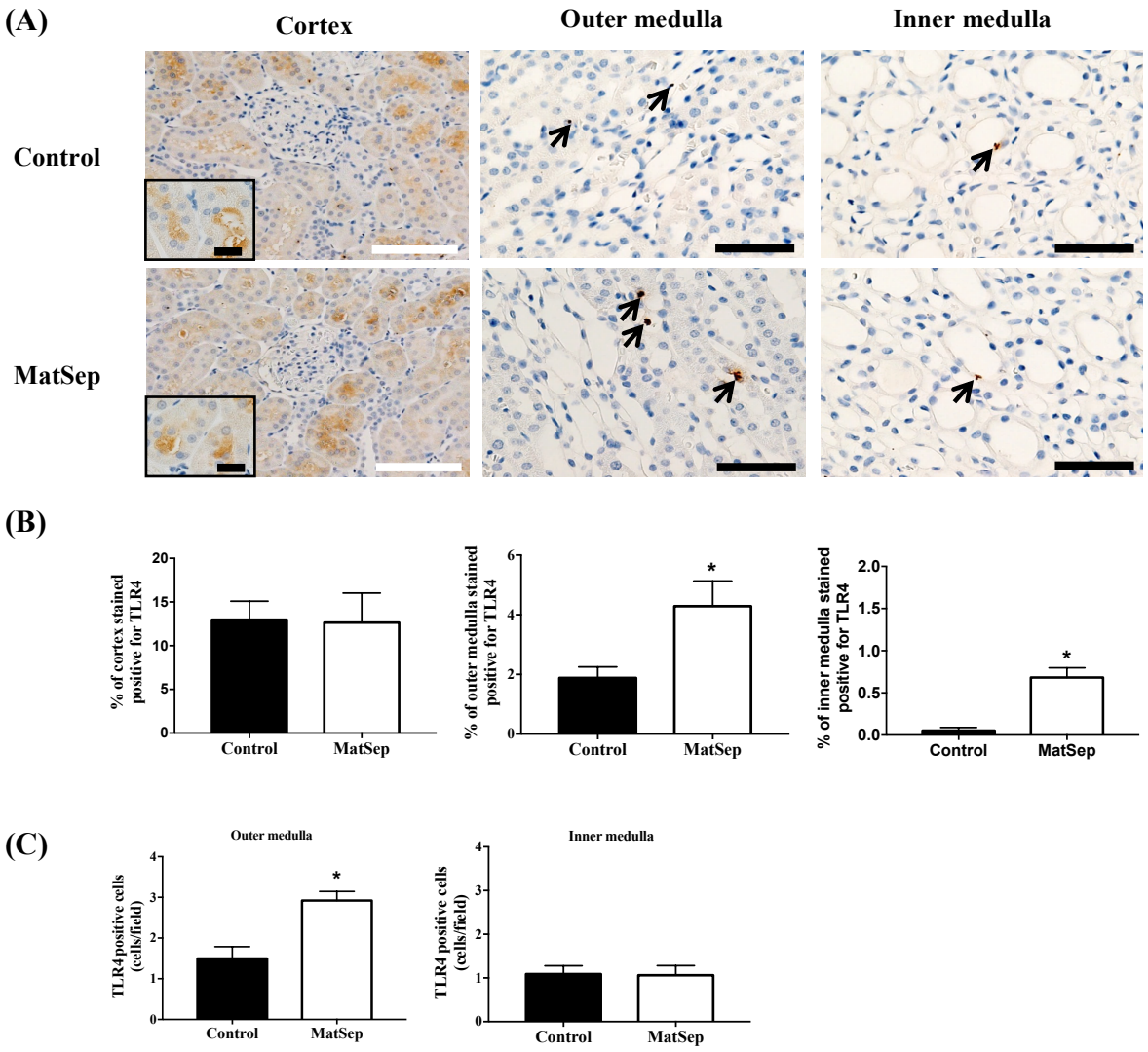


Figure 5. (A) Representative images of TLR4 in the renal cortex, outer medulla and inner medulla of control and MatSep rats. (B) Percentage of cortex, outer medulla, and inner medulla stained positive for TLR4. (C) Numbers of TLR4 positive cells in the outer medulla and inner medulla of control and MatSep rats. *$P < 0.05$ vs. control. n = 5-7/group. Black scale bar = 50μm; white scale bar = 20 μm; *inset* scale bar = 2mm.

MatSep is associated with increased cellular proliferation, increased numbers of activated neutrophils, and CD44$^+$ cells in the kidneys

Compared with the control group, MatSep rats displayed significantly increased cellular proliferation (as indicated by Ki-67 positive staining) in the outer medulla where the Ki-67 staining appears to be localized to the vasa recta (Figure 6). Ki-67 positive staining was observed within the cortical tubules and inner medullary interstitium yet was similar between control and MatSep rats (Figure 6).

To further examine the activation status of immune cells, we immunostained for myeloperoxidase, CD44, and CD69 positive cells. The number of activated neutrophils, as evidenced by myeloperoxidase (MPO) positive cells, was significantly higher in the renal cortex and outer medulla of MatSep rats compared to control rats (Figure 7). Image analysis of whole kidney scans demonstrated no significant difference in the percentage of CD44 positive staining present in cortex or outer medulla between the groups (Figure 8A-B); however, there was a trend in MatSep rats to show increased expression of CD44 in the outer medulla (Figure 8A-B). Upon further examination of the renal cortex at higher magnification, MatSep rats presented a greater percentage of glomeruli containing CD44$^+$ cells, as well as a greater number of CD44$^+$ cells/glomerulus than control rats (Figure 8C-D). Renal medullary CD44 positive staining was found in tubular localizations with similar staining in both controls and MatSep rats (Figure 8). CD69 positive cells were observed in both control and MatSep kidneys within distinct interstitial cells. We found no statistically significant difference in the number of CD69 positive cells in the renal cortex (control vs. MatSep, positive cells/renal cortex; 26.0 ±

6.58 vs. 34.22 ± 5.81; P = 0.37) or in the renal medulla (control vs. MatSep, positive cells/renal medulla; 18.83 ± 6.06 vs, 34.67 ± 6.92; P = 0.12) of control and MatSep rats.

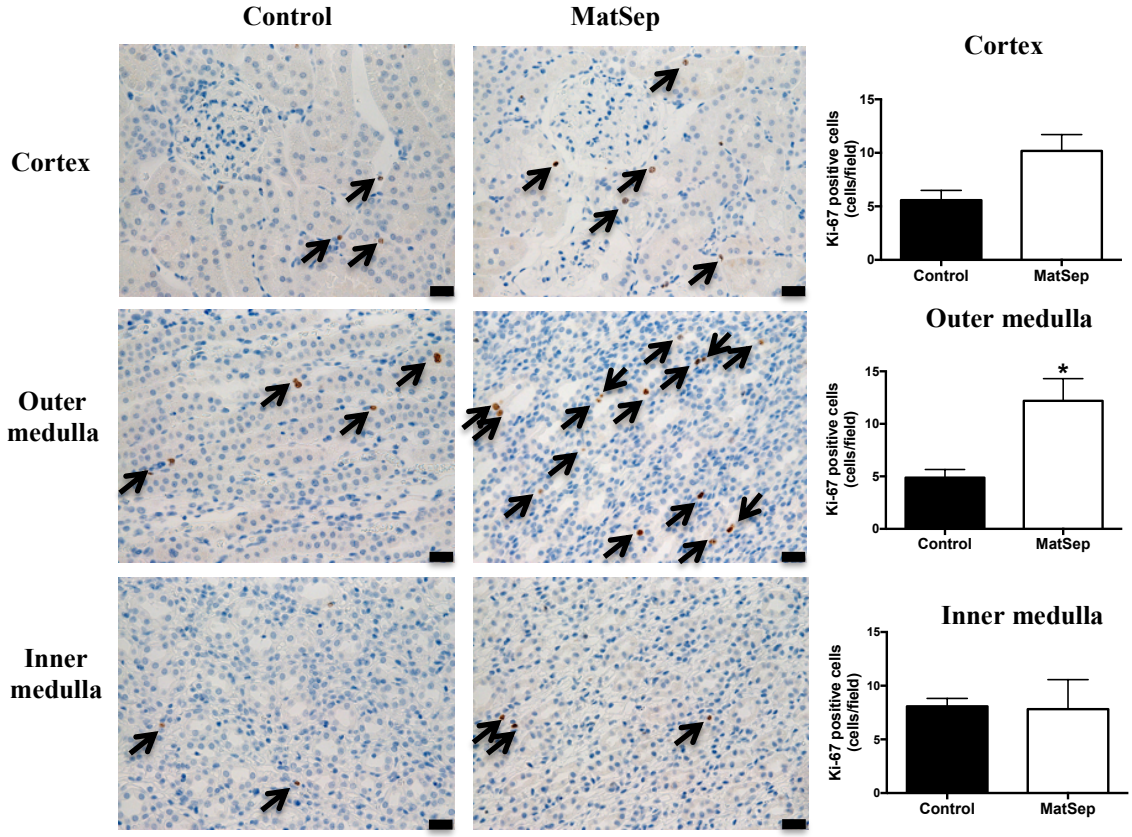


Figure 6. (A) Representative images of proliferating cells (Ki-67 positive cells) in the renal cortex, outer medulla, and inner medulla of control and MatSep rats. (B) Quantification of Ki-67 positive cells in cortex, outer medulla and inner medulla of control and MatSep rats. *$P < 0.05$ vs. control. n = 6-8/group. Scale bar = 20μm.

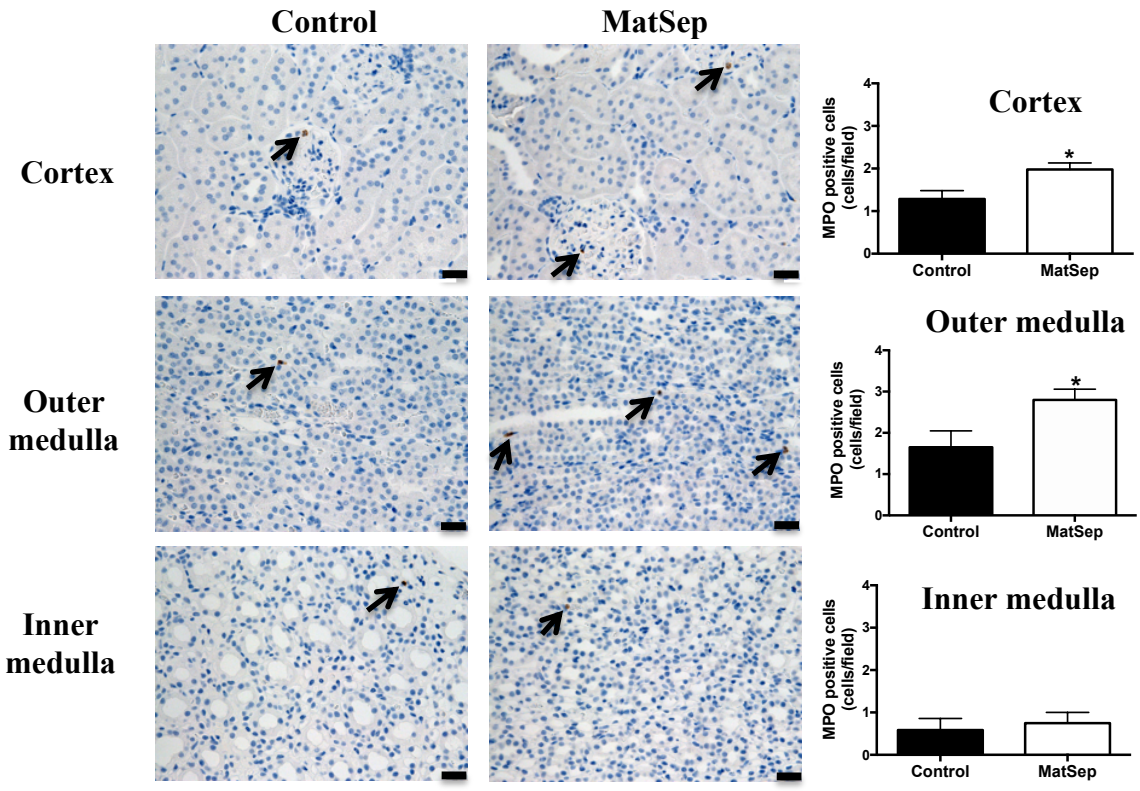


Figure 7. Representative images of activated neutrophils (MPO positive cells) in the renal cortex, outer medulla and inner medulla of control and MatSep rats. *$P < 0.05$ vs. control. n = 6-8/group. Scale bar = 20μm.

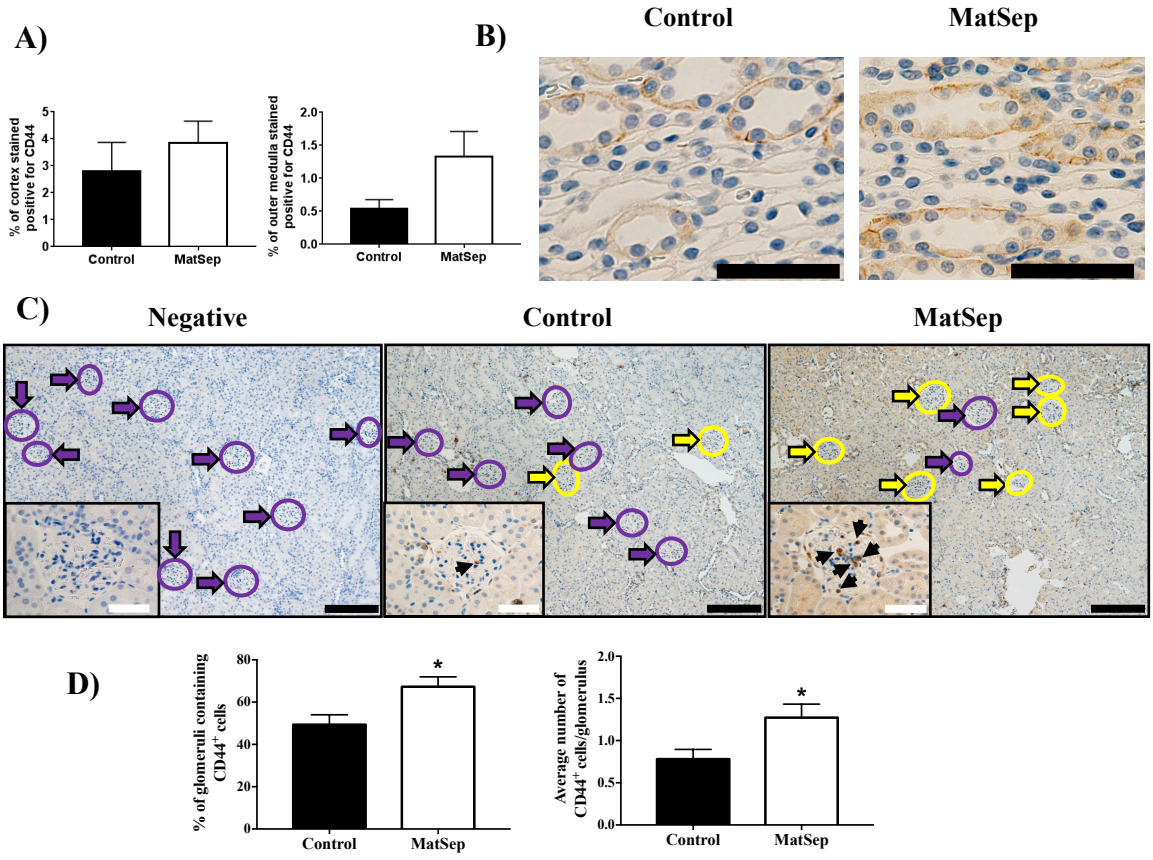


Figure 8. (A) Percentage of renal cortex or renal outer medulla stained positive for CD44. n=5-8/group. (B) Representative images of $CD44^+$ protein expression in renal outer medulla of control and MatSep rats. Scale bar = 50µm. (C) Representative images of CD44 staining in cortex from control and MatSep rats. Yellow circles and arrows indicate glomeruli containing $CD44^+$ cells, and purple circles and arrows indicate glomeruli with no $CD44^+$ cells. Black arrows in the inset photos designate $CD44^+$ cells within the glomerulus. Black scale bar = 100 µm; white scale bar = 20 µm. (D) Percentage of glomeruli containing $CD44^+$ cells and average number of $CD44^+$ cells/glomerulus in control and MatSep rat renal cortex. n = 5-8/group.

MatSep sensitizes rats to an immune challenge in adulthood

To determine if MatSep sensitizes rats to an immune challenge in adulthood, male MatSep and control rats were challenged with a single low dose (2 mg/kg i.v. injection) of LPS. Absolute numbers of mononuclear and T-cells in the blood and kidneys were similar in LPS treated control and MatSep rats (Figure 9).

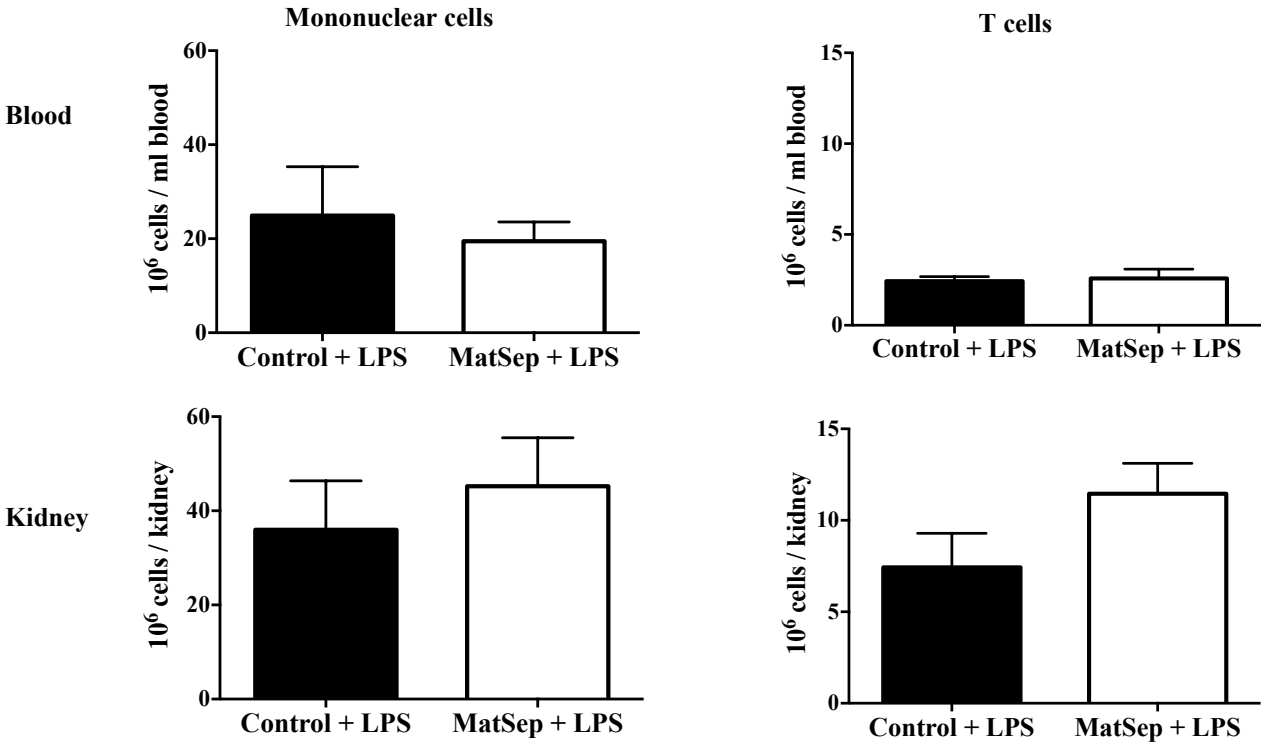


Figure 9. Absolute number of mononuclear and T cells in blood and kidney of control and MatSep rats after low dose LPS treatment. n = 5-7/group.

The changes in expression of 84 genes encoding inflammatory cytokines and receptors were analyzed in whole kidneys in the presence and absence of low dose LPS treatment. Low dose LPS treatment in control rats resulted in no changes in gene expression when compared to control rats without LPS treatment ($P>0.05$ for all 84 genes). On the contrary, the response to low dose LPS treatment in MatSep rats was more dramatic with 20 genes altered: 17 of the 84 genes were up-regulated, and 3 of the 84 genes were down-regulated (Table 2). To verify these array results, RT-PCR quantitative measurements were determined for C3, CXCL6, CXCL11, CXCL2, CXCL9, CCL12, CCL19, CXCL1, and CCL3. All the genes except CXCL6 were found to be significantly increased in MatSep rats with LPS treatment compared to MatSep rats treated with vehicle (Table 3). Surprisingly, no differences were observed in the abundance of renal IL-1β, IFN-γ, IL-6, or IL-4 in the kidneys of LPS-treated control vs. LPS-treated MatSep rats (Table 4).

MatSep is associated with decreased levels of IL-4 and IFN-γ in spleen

To further determine systemic levels and tissue specificity of cytokine production in control and MatSep rats, the levels of IL-1β, IL-4, IL-6, TNF-α, and IFN-γ in spleen, plasma and aorta were assessed. IL-4 and IFN-γ were significantly decreased in spleens from MatSep compared to control rats (Table 1), while IL-1β, IL-6 and TNF-α were similar between groups. In addition, IL-1β, IL-4, IL-6, IFN-γ, and TNF-α were below detection in the plasma and aorta of control and MatSep rats. LPS treatment did not induce differences in splenic levels of IL-1β, IL-4, IL-6, IFN-γ, and TNF-α (Table 4) in control and MatSep rats.

Table 2. Effect of acute LPS treatment on the relative mRNA expression of cytokine and receptor genes in whole kidney of MatSep (measured by PCR array)

Gene	Fold change	95% CI	p value
Complement component 3	46.86	(23.98, 88.44)	0.0159
Chemokine (C-X-C motif) ligand 6	45.54	(16.46, 89.44)	0.0159
Chemokine (C-X-C motif) ligand 11	21.78	(15.95, 26.24)	0.0286
Chemokine (C-X-C motif) ligand 2	14.64	(7.79, 25.05)	0.0357
Chemokine (C-X-C motif) ligand 9	12.51	(5.09, 16.14)	0.0159
Chemokine (C-C motif) ligand 12	10.91	(6.46, 19.13)	0.0357
Secreted phosphoprotein 1	7.90	(2.52, 17.16)	0.0159
Chemokine (C-C motif) receptor 1	7.68	(3.80, 10.20)	0.0159
Chemokine (C-C motif) ligand 19	4.58	(3.76, 5.83)	0.0286
Interleukin 1 beta	4.47	(1.78, 7.20)	0.0159
Chemokine (C-X-C motif) ligand 1	3.50	(1.92, 5.31)	0.0159
Chemokine (C-C motif) ligand 3	3.29	(1.04, 5.39)	0.0286
Integrin alpha M	3.16	(1.34, 4.50)	0.0159
Chemokine (C-X-C motif) ligand 10	2.17	(0.41, 4.68)	0.0317
Chemokine (C-C motif) ligand 7	1.80	(1.04, 3.01)	0.0286
Interleukin 1 receptor, type II	1.62	(0.01, 3.21)	0.0286
Interleukin 8 receptor, beta	0.93	(0.10, 2.15)	0.0317
Chemokine (C-C motif) receptor 9	-0.68	(-1.40, -0.15)	0.0357
Macrophage migration inhibitory factor	-0.65	(-1.13, -0.27)	0.0159
Interferon gamma	-0.45	(-0.82, -0.06)	0.0357

$P < 0.05$ vs. MatSep + vehicle. Non-shaded genes represent upregulated genes in response to LPS and shaded genes represent downregulated genes. n = 4-5/group

Table 3. Effect of acute LPS treatment on the relative mRNA expression of cytokine and receptor genes in whole kidney of MatSep (individual RT-PCR)

Gene	Fold change	95% CI	P value
C3	207	(27.72, 425.6)	0.0043*
CXCL6	76.7	(15.54, 133.1)	0.057
CXCL11	129	(14.72, 73.84)	0.036*
CXCL2	109	(4.56, 218.3)	0.029*
CXCL9	23.9	(3.52, 36.32)	0.0043*
CCL12	49.1	(3.03, 65.91)	0.029*
CCL19	61.0	(15.37, 120.1)	0.0022*
CXCL1	37.1	(19.95, 49.4)	<0.0095*
CCL3	12.3	(4.16, 21.16)	0.0095*

*$P < 0.05$ vs. MatSep + vehicle. n=4-6/group.

Table 4. Protein levels of IL-1β, IL-4, IL-6, TNF-α, and IFN-γ in whole kidneys and spleens of control and MatSep rats after treatment with low dose LPS.

Cytokine	Control + LPS (pg/mg protein ± SEM)	MatSep + LPS (pg/mg protein ± SEM)	P value
KIDNEY			
IL-1β	37.64 ± 2.06	36.42 ± 4.36	0.80
IL-4	1.75 ± 0.89	0.76 ± 0.27	0.33
IL-6	13.91 ± 4.45	14.17 ± 2.31	0.96
TNF-α	BD	BD	
IFN-γ	2.56 ± 0.89	1.62 ± 0.48	0.39
SPLEEN			
IL-1β	334.70 ± 55.85	292.2 ± 69.01	0.65
IL-4	0.36 ± 0.066	0.44 ± 0.068	0.45
IL-6	13.51 ± 5.56	8.46 ± 3.0	0.45
TNF-α	2.15 ± 0.25	1.35 ± 0.3	0.066
IFN-γ	30.22 ± 6.35	29.11 ± 5.57	0.90

***$P < 0.05$ vs. control. n = 5-7/group.**

AIM 2: To test the hypothesis that in MatSep rats, LNAME activates renal CD8-positive T cells leading to exaggerated elevation in blood pressure.

Renal MatSep $CD8^+$ T cell is elevated in response to LNAME treatment

Findings from aim 1 indicates that MatSep renal immune cells are primed towards an inflammatory state; therefore, we hypothesized that in response to a hypertensive treatment there would be significant increase in MatSep immune cells compare to control immune cells. We focused on T cells because it is well documented that T cells are significantly increased during hypertension and our preliminary data (n=4/group) showed that the percentage of CD8 T cells, but not CD4 T cells, was elevated in MatSep rats after LNAME treatment. To further determine the role of CD8 T cells in LNAME induced hypertension, we increased our sample size and assessed the percentages of $CD8^+$ and $CD4^+$ T cells in control and MatSep kidneys in the presence and absence of LNAME. Data obtained by flow cytometry showed that renal MatSep $CD3^+CD8^+$ T cells were elevated, although not significantly with LNAME treatment (n=10-11/group in the LNAME groups) Figure 10A. No trend towards significance was observed for $CD3^+CD4^+$ T cells Figure 10B.

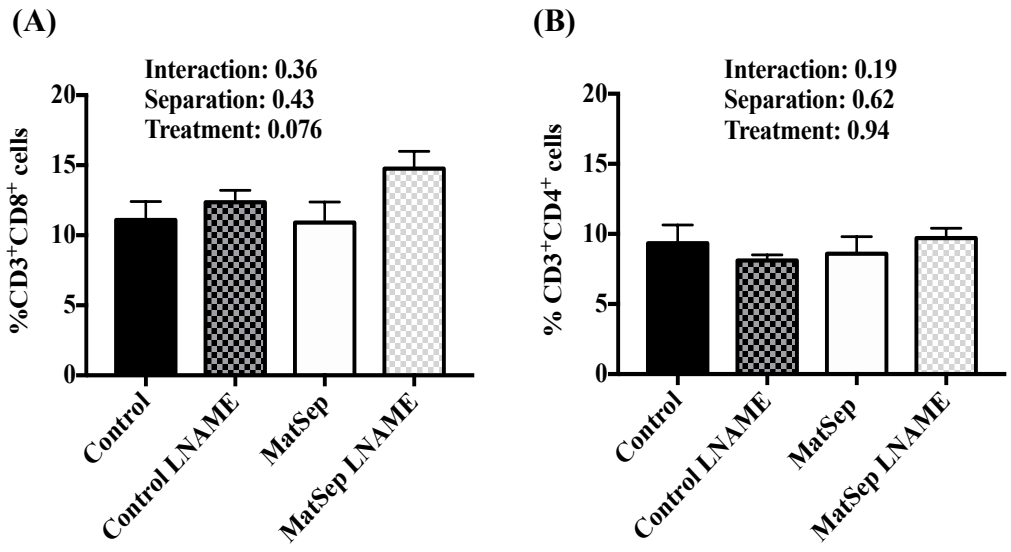


Figure 10. Percent gated event of T cells (A) CD8 T cells. (B) CD4 T cells. Statistical significance is determined as $P < 0.05$. n=4-11/group.

Urinary KIM 1 excretion is significantly decreased in MatSep rats in response to LNAME treatment

The presence of increased level of T cells in the kidneys is linked to renal damage. We then determined the effect of MatSep on glomerular and tubular damage in the presence and absence of LNAME. We assessed glomerular damage by measuring urinary albumin and nephrin excretion, while tubular damage was assessed by urinary KIM1 and NGAL excretion by ELISA. Urinary KIM 1 excretion was significantly increased in control and MatSep groups in response to LNAME compared to their respective baseline groups Figure 11C. However, within the LNAME treatment groups MatSep urinary KIM1 excretion is significantly lower than control Figure 11C. No

significant differences exist for urinary albumin, nephrin, and NGAL excretion Figure 11A, B, and D.

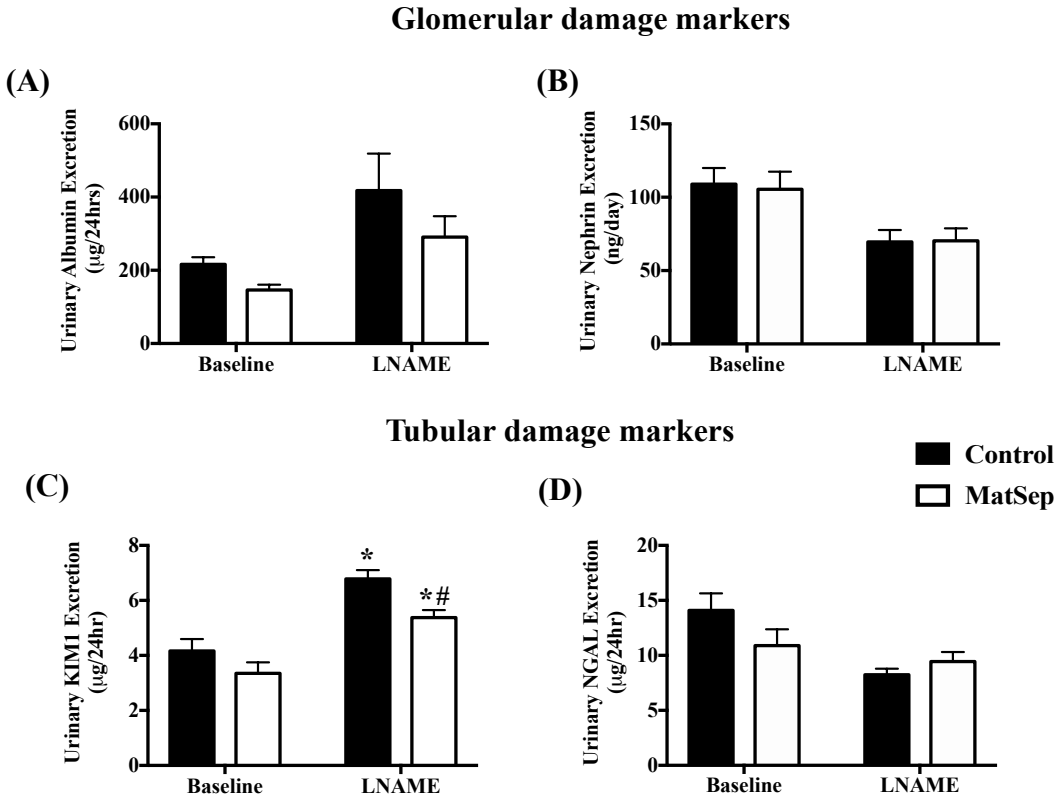


Figure 11. Effect of LNAME on urinary renal damage markers in control and MatSep rats. (A) Albumin excretion. (B) Nephrin excretion. (C) KIM1 excretion. (D) NGAL excretion. * represents $P < 0.05$ vs. baseline groups; # represents $P < 0.05$ vs. LNAME treated group. n=6-7/group.

Blood pressure is significantly lower in MatSep rats in the presence and absence of LNAME

Blood pressure parameters were measured by telemetry to determine whether differences exist between control and MatSep rats in the presence and absence of LNAME. In the absence of LNAME, MAP, SBP, and DBP were significantly lower in MatSep rats compared to control rats. The same difference was observed for the duration of LNAME treatment Figure 12A-C. However, heart rate was similar between control and MatSep rats in the presence and absence of LNAME.

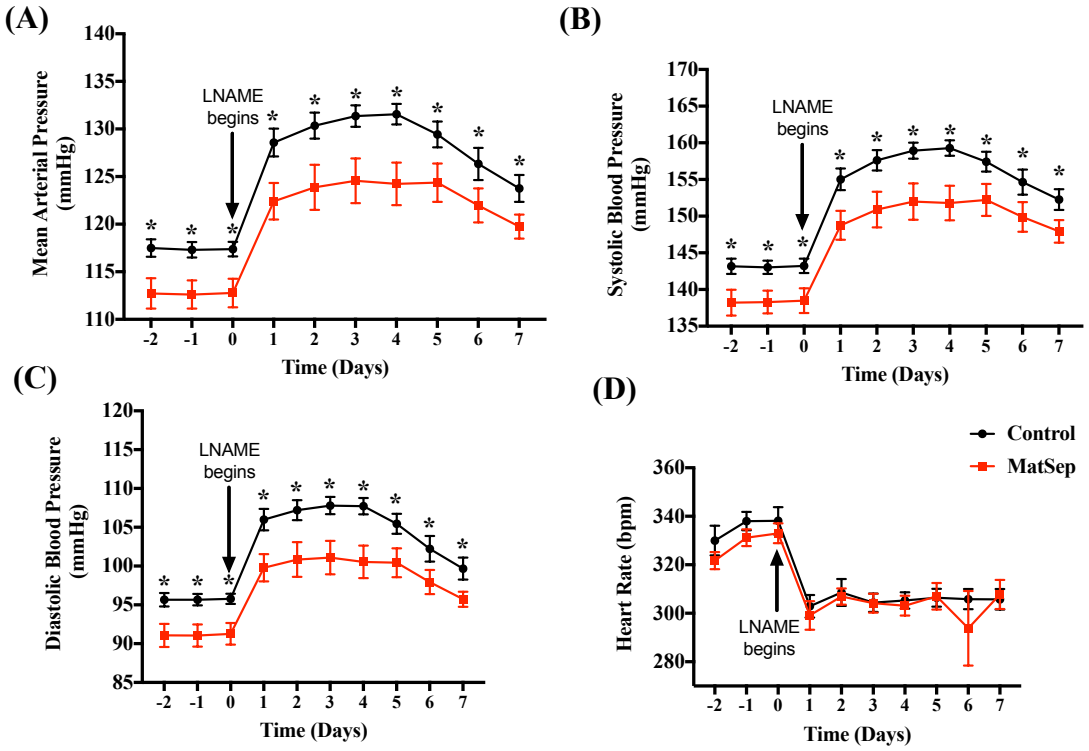


Figure 12. Blood pressure responses to LNAME treatment expressed as 24-hour averages. (A) mean arterial pressure (MAP). (B) Systolic blood pressure (SBP). (C) Diastolic blood pressure (DBP). (D) Heart rate (HR). * $P < 0.05$ vs. MatSep group at the same timepoint. n=8/group.

CHAPTER 5

DISCUSSION

Human studies have reported ELS as an emerging risk factor for CVD (Alastalo et al., 2012; Alastalo et al., 2013; Su et al., 2014; Su et al., 2015). An association between ELS and inflammation has also been observed in adults (Danese, Pariante, Caspi, Taylor & Poulton, 2007). The primary aim 1 was to characterize the renal inflammatory state in adult male MatSep rats. The hypothesis guiding this study was that MatSep induces renal priming of the immune response. We utilized a rat model of ELS, maternal separation (MatSep), to identify ELS-specific inflammatory mediators and/or pathways in adulthood. The main findings of these studies demonstrate that, when compared to control rats, MatSep rats display: 1) elevated IL-1β mRNA expression in circulating T cells as well as increased renal levels of IL-1β abundance, particularly localized in distal tubules, 2) increased TLR4 immunopositive interstitial cells in the renal medulla, 3) increased cellular proliferation in the renal medulla, 4) increased renal neutrophil activation, 5) greater numbers of glomerular CD44 immunopositive cells, and, 6) heightened renal cytokine and chemokine gene expression in response to LPS treatment. We also found that MatSep and control rats have similar numbers of circulating and renal mononuclear cells, T cells, and T cell subsets, while the population of circulating B cell numbers was significantly decreased in MatSep rats. Our results indicate that MatSep

leads to “priming” or sensitization of the immune system, resulting in an exaggerated gene expression response to an immune challenge in adulthood. This ELS-mediated sensitization of the immune system may play an important role in promoting cardiovascular disease earlier and more robustly in adulthood.

The present study showed that MatSep rats specifically express higher levels of IL-1β in the kidney compared to control rats. Macrophages and epithelial cells produce IL-1β, and it plays a critical role in modulating tubular transport and inflammatory responses of both the innate and adaptive immune response through activation of the IL-1 receptor (IL-1R) (Ben-Sasson et al., 2009; Nakamura, Hayashi & Kubokawa, 2015). Zhang et al (Zhang et al., 2016) recently published that the renal IL-1β/IL-1R pathway plays a role in the development of angiotensin II-induced hypertension. This study demonstrated that activation of the IL-1R prevents maturation of macrophages and blunts the induction of nitric oxide (NO), thereby limiting the NO-dependent natriuresis *via* the sodium-potassium-two-chloride (NKCC2) transporter. Our laboratory previously published that MatSep induces a NO deficient phenotype (Loria, Kang, Pollock & Pollock, 2011). We propose that the elevated IL-1β levels observed in the distal tubule from MatSep rats may be related to the NO deficient phenotype. Further research is needed to understand whether these pathways are linked in the MatSep model. To begin to understand possible pathways linking ELS to the IL-1β pathway, we examined localization and abundance of TLR4 in the kidneys of control and MatSep rats. We found an approximate two-fold increase in the number of TLR4 immunopositive cells specifically in renal medullary interstitial cells in MatSep rats. Activation of TLR4 is known to mediate increased expression of a variety of pro-inflammatory cytokines

including IL-1β (Grishman, White & Savani, 2012). Souza et al (Souza et al., 2015) recently showed that TLR4 deficiency in renal epithelial cells led to decreased IL-1β expression *in vitro*. However, further studies are needed to determine whether there is a direct or indirect link between TLR4 signaling and IL-1β in renal tubules of MatSep rats. These observations suggest that MatSep primes the innate immune system within the kidney.

Human studies revealed that chronic psychosocial stress such as depression is associated with increased numbers of circulating neutrophils (Maes et al., 1992). Blood isolated from depressed and non-depressed patients were analyzed for differences in myeloperoxidase (MPO) content. The results showed increased numbers of circulating neutrophils, elevated circulating monocytes and total number of white blood cell count (Maes et al., 1992). Since MatSep is a well-studied model for depressive-like behaviors, we assessed activation of neutrophils by MPO positive cells within the kidney (Papayannopoulos, Metzler, Hakkim & Zychlinsky, 2010). We found a significant increase in MPO positive cells in MatSep cortex and outer medulla compared to control kidneys, suggesting that ELS leads to a pro-inflammatory state in adult rat kidneys. Neutrophils are mainly activated by chemokine and cytokines(Wright, Moots, Bucknall & Edwards, 2010). The increased IL-1β in the renal tubular epithelial cells and the up-regulation of several cytokines and chemokines may contribute to the MatSep-specific neutrophil activation. Furthermore, we also assessed cellular proliferation by staining for Ki-67 and found a significant elevation specifically in renal outer medulla. Upon close observation, the proliferating cells appear to be localized to the vasa recta although future studies with specific markers are necessary to verify this initial observation.

Nevertheless, MatSep is associated with increased cellular proliferation as well as increased activation of neutrophils and, taken together with the elevated abundance of IL-1β and increased TLR4 immunopositive cells, indicates a phenotype with activation of the innate immune system. Taken together, all of these observations suggest that MatSep-induced specific changes may be more specific to the outer medullary region of the kidney, which is a region known to be sensitive to renal injury (El Sabbahy & Vaidya, 2011).

CD44 is a glycoprotein expressed on the surface of endothelial cells, leukocytes, epithelial cells, keratinocytes and fibroblasts and also considered the primary receptor for hyaluronan. CD44 is important in physiological functions including cellular proliferation, lymphocyte activation, hematopoiesis, cell adhesion and migration (Ponta, Sherman & Herrlich, 2003). Furthermore, various studies have implicated CD44 in renal disease, vascular disease, bacterial infection, liver disease, and in wound healing (Jordan, Racine, Henning & Lokeshwar, 2015; Patouraux et al., 2017). In rodent models of renal disease, increased expression of CD44 resulted in worsening of disease. For example, in an experimental rat model of crescentic glomerulonephritis, CD44 protein expression was significantly increased in infiltrating macrophages within the glomerular crescents (Jun et al., 1997). Additionally, migrating leukocytes that adhered to the endothelium also had high expression of CD44 compared to healthy rats (Jun et al., 1997). This observation correlated with significant increases in proteinuria, serum creatinine, and creatinine clearance in the diseased rats (Jun et al., 1997). The involvement of CD44 in renal disease has also been reported in humans. Renal biopsy from patients with acute renal allograft rejection showed significant increase in CD44 expression in the tubules and

infiltrating cells compared to non-rejecting renal biopsies (Rouschop et al., 2006). In a separate study, in biopsies of patients with severe renal histological damage, CD44 expression was significantly higher in interstitial infiltrates and in infiltrating cells in the glomerulus (Roy-Chaudhury et al., 1996). In line with these studies, we report a significant increase in CD44+ cells in glomeruli from MatSep rats compared to control rats, suggesting activation of immune function. Future studies are needed to determine which population of cells expressed CD44 in MatSep glomerulus and whether it affects renal function. Conversely, we stained for the presence of CD69 in control and MatSep kidneys. Unlike CD44, CD69 is an early T cell activation marker. It is a type II C-lectin membrane receptor expressed at low levels in naïve T cells (Gonzalez-Amaro, Cortes, Sanchez-Madrid & Martin, 2013). Upon T cell activation, CD69 becomes highly expressed leading to inflammatory responses (Gonzalez-Amaro, Cortes, Sanchez-Madrid & Martin, 2013). In this study, we found no differences in this activation marker in MatSep rats compared to control rats, suggesting that MatSep alone most likely does not activate T cells and the adaptive immune system. Although further studies are necessary to fully characterize the T cell activation status in MatSep rats.

We also assessed protein levels of pro-inflammatory cytokines in the plasma, aorta, and spleen in order to determine whether the observed cytokine differences between control and MatSep rat kidneys are similar in other tissues or specific to the kidney. Blood vessels participate in inflammatory processes by up-regulating expression of cytokines, chemokines, and adhesion molecules to allow extravasation of immune cells into the site of inflammation (Zgraggen, Ochsenbein & Detmar, 2013). IL-1β, IL-4, IL-6, IFN-γ, and TNF-α were below the detection limit of the assay in the plasma and

aorta of control and MatSep rats suggesting that MatSep does not elicit a systemic immune response. These findings are interesting because they suggest that the immune programming due to MatSep may be localized to organs. The spleen serves as a reservoir for immune cells and a site for antigen presentation allowing for the release and homing of immune cells to inflammatory sites (Bronte & Pittet, 2013). In the spleen, we observed that IL-4 and IFN-γ were significantly lower in MatSep rats than in control rats suggesting MatSep induces distinct cytokine profiles in the kidney and spleen.

Finally, we assessed whether an immune challenge results in differential expression of chemokines/cytokines and their receptors in the kidney of MatSep and control rats. Low dose LPS treatment showed significant up-regulation of 17 genes and down-regulation of 3 genes in the MatSep rats (Table 1), while low dose LPS treatment resulted in no significant changes in gene expression in control rats ($P>0.05$). LPS is a known ligand for TLR4, thus we propose that the MatSep-induced changes in chemokine expression are most likely TLR4-dependent. Chemokines are a group of cytokines with chemotactic properties produced by a variety of cell types and play an important role in recruiting immune cells to the site of an immune response (Griffith, Sokol & Luster, 2014). Tissue expression of specific chemokine or chemokine receptors drive distinct immune cell recruitment and response (Mantovani, 1999). Complement component 3 (C3) showed the highest expression with LPS treatment in MatSep rats. C3 is involved in a host of inflammatory kidney diseases including hereditary renal diseases (Koscielsk-Kasprzak, Bartoszek, Myszka, Zabinska & Klinger, 2014). A number of ligands for C-X-C motif (CXCL) were also highly expressed in response to LPS in MatSep rats. CXCL2 and CXCL6 are neutrophil-recruiting chemoattractants (De Filippo et al., 2013; Jovic et

al., 2016), while CXCL9 is implicated in crescentric glomerulonephritis (Richard et al., 2008). CXCL11 and CXCL9 are ligands for CXCR3 (Groom & Luster, 2011). The expression of CXCR3 in T cells allows for T cell differentiation and infiltration into injured tissues(Groom & Luster, 2011). CXCL1, chemokine motif ligand (CCL) CCL3, CCL19 and CCL12 are involved in recruitment of inflammatory cells in renal diseases as seen in ischemia reperfusion injury (Furuichi, Gao, Horuk, Wada, Kaneko & Murphy, 2008; Guo et al., 2016; Miura, Fu, Zhang, Remick & Fairchild, 2001) and kidney allograft rejection rodent models (Ziegler et al., 2006). Additionally, interleukins are produced by leukocytes and endothelial cells participating in regulating immune cell differentiation (Akdis et al., 2011). The upregulation of chemokines and cytokines that we found in kidneys of MatSep rats in response to LPS supports the hypothesis that ELS primes the immune system.

These data indicate that MatSep induces programming of the innate immune system, especially the TLR4 and IL-1β pathways. Several possible mechanisms exist linking MatSep to the priming of the innate immune system that may be relevant for discussion. Our laboratory previously showed that MatSep induces sensitization of the renal and systemic sympathetic nervous system (SNS) (Loria, Brands, Pollock & Pollock, 2013). Several recent publications show a link between the SNS and the innate immune response (Powell et al., 2013; Scanzano & Cosentino, 2015). We also previously found that MatSep down-regulates endothelin receptor expression (Loria, D'Angelo, Pollock & Pollock, 2010) and it is well-accepted that the endothelin system regulates immune responses (Kohan & Barton, 2014; Saleh, 2010; Saleh, 2011) although few details are understood about specific mediators or cell types. Furthermore, MatSep is known to

increase activation of the hypothalamic-pituitary-adrenal (HPA) axis during early life (Lippmann, Bress, Nemeroff, Plotsky & Monteggia, 2007; Plotsky, 1993). The HPA axis is a known modulator of immune function (Bellavance & Rivest, 2014). We propose that MatSep-induced modulation of TLR4, IL-1β, SNS sensitivity, endothelin system, and/or the HPA axis during early life re-programs and primes the renal immune system.

The purpose of aim 2 is to determine how MatSep CD8 T cells respond to LNAME. So far, we found that (1) $CD8^+$ T cells are higher in MatSep rat kidneys although not statistically significant, (2) in response to LNAME, urinary KIM1 excretion is significantly lower in MatSep rats compared to control rats, and (3) MAP, SBP, and DBP is significantly lower at baseline and in response to LNAME in MatSep compared to control rats.

NO has both protective and damaging effects during pathophysiological conditions. In a mice model of shock syndrome induced by bacterial staphylococcal enterotoxin B (SEB), administration of SEB induced production of NO as measured by increased serum levels of nitrate and nitrite, and these increases in nitrate and nitrate was mediated by TNF-α, and IFN-γ. In-vivo and in-vitro studies revealed that NO inhibition using LNAME increased TNF-α, and IFN-γ in the serum and by splenocytes respectively in response to SEB. Interestingly, the enhanced cytokine production in mice given SEB and treated with LNAME led to a 95% mortality rate compared to mice that received SEB or LNAME alone; hence the protective role of NO during SEB infection (Florquin, Amraoui, Dubois, Decuyper & Goldman, 1994). Additionally, in response to SEB treatment, iNOS (NOS2) was significantly increased in $CD3^+$ T cells and inhibition of iNOS activity by $_L$-NIL led to significantly increased serum levels of TNF-α and IFN-γ,

and delayed intestinal ion transport post treatment with SEB, an indication that NO is important in intestinal recovery (McKay, Lu, Jedrzkiewicz, Ho & Sharkey, 1999). Conversely, another study examined the role of IL-12, IFN-γ, lymphotoxin-alpha, and NO during staphylococcus aureus endotoxin. Their findings revealed NO is important for lymphotoxin production (Sriskandan, Evans & Cohen, 1996). These studies did not specifically examine any specific immune cells.

NO regulation of T cells has been studied. In NOSII knockout mice, there was increased Th17 cell differentiation, and NOS2 expression in $CD4^+$ T cells led to CD4 T cells activation and increased proportion of IL-17 producing $CD4^+$ T cells. When T cells from NOS2 knockout mice were cultured in NO donors, IL-17 production was significantly decreased in a dose dependent manner, an indication of a protective role (Xue, Yan, Zhang & Xiong, 2018; Yang et al., 2013). $CD8^+$ T cells significantly increased differentiation into Th1 cells and IFN-γ production at low concentration of NO donor (10μM) in vitro but not at high concentration of NO donor (100μM) (Niedbala, Cai & Liew, 2006). These pro and anti-inflammatory effects of NO is dependent on the source of NO, susceptibility of the immune cells, NO concentration, and the redox environment; as a result, there is difficulty in properly establishing the role of NO during pathological conditions (Ibiza & Serrador, 2008). In our study we showed that LNAME may increase the proportion of $CD8^+$ cells but not CD4 T cells. Further studies are needed to understand the involvement and physiological implication, if any, of CD8 T cells in LNAME treated MatSep rats. NO also regulates macrophage polarization and can modulate the action of other innate immune cells. Even though this study only focused on

T cells, it would also be important to determine how NO blockade affects innate immunity in MatSep rats.

In the cardiovascular system, NO is an important in maintaining vascular function by mediating the relaxation of vascular smooth muscles, inhibition of platelet aggregation and adhesion, prevention of pro-inflammatory and pro-proliferative effects of leucocytes, thereby elevating blood pressure (Hermann, Flammer & Luscher, 2006). Genetic deletion of NOS3 isoform has elevated blood pressure phenotype and lower heart rate, thus NOS3 is important in maintaining blood pressure and heart rate (Shesely et al., 1996). Other studies have reported that LNAME treatment leads to development of hypertension (Arnal, El Amrani, Chatellier, Menard & Michel, 1993; Gardiner, Kemp, Bennett, Palmer & Moncada, 1992; Ribeiro, Antunes, de Nucci, Lovisolo & Zatz, 1992)

Previous studies from our laboratory showed that pre-incubation of intact aortic rings with LNAME induced greater vasoconstriction to AngII in control rats than in MatSep rats, however, the maximal vasoconstriction to LNAME was greater in MatSep rats than in control rats. Blood pressure measurements showed a greater blood pressure response to AngII in MatSep rats compare to controls, but treatment with LNAME during the second week of AngII treatment showed no further increase in blood pressure in MatSep rats (Loria, Kang, Pollock & Pollock, 2011). Further blood pressure elevation was observed in control rats when treated with LNAME. Significant elevation in heart rate was also observed in AngII treated control rats during LNAME exposure compared to MatSep rats. Interestingly, similar NOS, NOS1, NOS2, and NOS3 enzymatic activities was observed in control and MatSep rats. NOS1 and NOS3 aortic protein expression were similar as well (Loria, Kang, Pollock & Pollock, 2011). These findings suggest a

MatSep led to decrease in NOS buffering capacity and differences in cardiac NOS pathways independent of any influence in NOS activity or NOS isoform expression.

Surprisingly, in this study we see a significant lower blood pressure in MatSep rats compared to control rats at baseline and in response to chronic LNAME treatment even though LNAME led to elevated blood pressures in both groups. In previous studies, we did not observe any baseline differences in blood pressure (Loria, Kang, Pollock & Pollock, 2011; Loria, Pollock & Pollock, 2010; Loria, Yamamoto, Pollock & Pollock, 2013). Our findings suggest that there may be changes in the central nervous system that, at least in part, explain the observed blood pressure differences in MatSep rats at baseline which is maintained in response to chronic LNAME.

NO interacts with the endothelin and the sympathetic nervous systems. In the kidneys, the endothelin system stimulates NO production in the collecting ducts (Hyndman, MacDonell & Pollock, 2012). ET1 binding to the ETB receptor increases NO production without changing NOS1 and NOS2 protein expression in the kidney, resulting in sodium excretion via inhibition of the sodium (ENaC) channel (Hyndman & Pollock, 2014). Plasma ET1 is significantly higher in MatSep rats at baseline compared to control rats and significantly low aortic ETA and ETB receptor expression (Loria, D'Angelo, Pollock & Pollock, 2010). Additional studies are needed to determine whether LNAME induced hypertension alters ET1, ETA, and ETB expressions in MatSep rats. Furthermore, NO acts as a neurotransmitter in the brain to alter the sympathetic nervous system and in turn affect blood pressure to mediate hypertension. Previous studies in MatSep rats showed baseline elevation of norepinephrine (NE) in the kidneys, aorta, spleen, adrenals, and left ventricles. No brain measurements of NE were conducted in

MatSep rats or in response to LNAME. To fully understand the observed blood pressure differences in control and MatSep rats at baseline and in response to LNAME, future studies would determine the role of NO at baseline and in response to NO blockade in the brain, kidneys and blood vessels, the interaction between NO and the central nervous system in controlling blood pressure, and the role of NO and it's interaction with the ET system in MatSep rats.

Lastly, glomerular and tubular injury are markers of renal damage in hypertension. Our study showed that urinary KIM 1 damage was significantly lower in MatSep rats compared to control rats an indication that MatSep rats are protected from LNAME induced tubular damage. Again, further studies are needed to determine the mechanism by which MatSep is protected from renal tubular damage in LNAME induced hypertension, and the involvement of immune cells.

In conclusion, this study utilized an animal model of ELS to support the plausibility of the positive associations between ACE and inflammation in humans. Characterization studies confirmed that MatSep kidneys were primed and are indeed in an inflammatory state, suggesting that renal MatSep immune cells may play a role in blood pressure regulation. The second part of the study then aimed to understand how MatSep rats responds to LNAME-induced hypertension. Very surprisingly, blood pressure response was significantly reduced in MatSep rats and the kidneys were protected against renal tubular injury. Furthermore, CD8 T cells were slightly higher in MatSep rats in response to LNAME. Future studies will be designed to understand how NO influences renal immune cells responses, renal damage, and blood pressure regulation in MatSep rats.

CHAPTER 6

PERSPECTIVE

ELS is now an established risk factor in developing CVD and hypertension. With the immune cells being a known mechanism for developing and maintaining hypertension, this study is important because characterization of the inflammatory status in MatSep rats makes it a useful model for studying how MatSep primes renal immune cells, and how those immune cells affect blood pressure responses during a secondary insult.

Findings from the second aim of this study suggests that MatSep rats have significantly lower baseline blood pressure in response to LNAME-induced hypertension compared to control rats, are protected against LNAME-induced renal tubular damage, and have a higher renal $CD8^+$ T cells. Our hypothesis hinges on the role that renal $CD8^+$ T cells play in mediating the differences in blood pressure response between control and MatSep rats; as such, more work needs to be done to determine the direct role of $CD8^+$ T cells.

Additional experiments

AIM 2b: To determine if renal $CD8^+$ T cells are activated in MatSep rats compared to control rats in response to LNAME.

While this study reported a higher proportion of renal $CD8^+$ T cells in MatSep rats, the activation state of the $CD8^+$ T cells are unknown. To determine the activation state of renal $CD8^+$ T cells, the proportion of $CD8^+$ T cells-producing IFN-γ, IL-17, and TNF-α in response to LNAME need be assessed by flow cytometry. Alternatively, renal $CD8^+$ T cells can also be isolated from control and MatSep rats using magnetic beads. The cells are then treated with LNAME and the concentration of IFN-γ, IL-17, and TNF-α is determined by ELISA. Furthermore, cytokines produced by $CD8^+$ T cells are capable of causing renal damage and sodium retention by stimulating renal sodium transporters (Liu et al., 2017). Immunoblotting of control and MatSep kidney homogenates in the presence and absence of LNAME would show any changes in protein expression of NKCC transporter and NC channel in the thick ascending limb and distal convoluted tubule respectively. Histological changes in the kidneys after treatment with LNAME can also be visualized by assessing renal fibrosis with Masson Trichrome stain. Based on the findings so far, it is expected that IFN-γ, IL-17, and TNF-α production by MatSep rats would be less compared to control rats. Additionally, it is expected that sodium transporter and channel expression would be less in MatSep rats, and MatSep kidneys would be protected against renal fibrosis compared to controls.

AIM 2c: To determine if blocking $CD8^+$ T cells would lower LNAME- induced blood pressure elevation in MatSep rats.

To directly test the effect of blocking $CD8^+$ T cells on blood pressure, deplete CD8 T cells by treating MatSep rats with anti CD8 monoclonal antibody intravenously (Huang, Macary, Kemeny & Chung, 1999) prior to LNAME exposure, then give LNAME to observe any changes in blood pressure responses. Alternatively, adoptive transfer is another useful technique for determining the involvement of $CD8^+$ T cells in mediating blood pressure responses observed in MatSep rats in response to LNAME. This technique would involve isolating splenic $CD8^+$ T cells from MatSep rats that have not been treated with LNAME, then injecting those $CD8^+$ T cells into control rats before treating the control rats with LNAME. MatSep rats treated with LNAME should have the same blood pressure phenotype as LNAME treated control rats which received MatSep CD8 T cells. Additional parameters such as glomerular and renal tubular damage, renal fibrosis, sodium transporters and channel protein expressions, and renal concentrations of IFN-γ, IL-17, and TNF-α can be assessed as well. The results from assessing these parameters would be similar to the anticipated results in aim 2b.

The field of immune-mediated CVD induced by ELS in rodent models is still in its infancy. It is also important to note that the studies presented here were all conducted in males. Our understanding of molecular mechanisms through which inflammation might lead to CVD development is still very limited. The few studies from rodent models have shed light on potential mechanisms that might mediate the inflammation in humans

(Figure 2). These mechanisms involve catecholamines, reactive oxygen species, nitric oxide, and dysregulation of the endothelin system. Future studies on the role of these mechanisms in immune cell responses that arise in rodent models of ELS would allow us to understand how early life adversity contributes to CVD in adulthood.

The long-term goal in this field is that by deciphering the immune mediated mechanisms of early life adversity and sex differences associated with these mechanisms, it would allow for targeted and personalized therapies in males and females who are diagnosed with CVD. Secondly, health care providers can use the awareness of past childhood adversity to educate patients of their susceptibility to CVD, and healthcare providers can also suggest prevention strategies, such as lifestyle and dietary changes that would help alleviate the severity and burden of disease outcomes in those patients.

REFERENCES

1. Adler HS, Simon A, Graulich E, Habermeier A, Bacher N, Friebe A*, et al.* (2010). Neuronal nitric oxide synthase modulates maturation of human dendritic cells. J Immunol 184**:** 6025-6034.

2. Agata J, Masuda A, Takada M, Higashiura K, Murakami H, Miyazaki Y*, et al.* (1997). High Plasma Immunoreactive Leptin Level in Essential Hypertension. Am J Hypertens 10**:** 1171-1174.

3. Akdis M, Burgler S, Crameri R, Eiwegger T, Fujita H, Gomez E*, et al.* (2011). Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. J Allergy Clin Immunol 127**:** 701-721.e701-770.

4. Alastalo H, Raikkonen K, Pesonen AK, Osmond C, Barker DJ, Heinonen K*, et al.* (2012). Cardiovascular morbidity and mortality in Finnish men and women separated temporarily from their parents in childhood--a life course study. Psychosom Med 74**:** 583-587.

5. Alastalo H, Raikkonen K, Pesonen AK, Osmond C, Barker DJ, Kajantie E*, et al.* (2009). Cardiovascular health of Finnish war evacuees 60 years later. Ann Med 41**:** 66-72.

6. Alastalo H, Räikkönen K, Pesonen AK, Osmond C, Barker DJP, Heinonen K*, et al.* (2013). Early life stress and blood pressure levels in late adulthood. J Hum Hypertens 27**:** 90-94.

7. Amador CA, Barrientos V, Pena J, Herrada AA, Gonzalez M, Valdes S*, et al.* (2014). Spironolactone Decreases DOCA–Salt–Induced Organ Damage by Blocking the Activation of T Helper 17 and the Downregulation of Regulatory T Lymphocytes. Hypertension 63**:** 797-803.

8. American Diabetes Association (2018). Economic cost of diabetes in the U.S in 2017. Diabetes Care 41**:** 917-928.

9. Amini-Khoei H, Mohammadi-Asl A, Amiri S, Hosseini MJ, Momeny M, Hassanipour M*, et al.* (2017). Oxytocin mitigated the depressive-like behaviors of maternal separation stress through modulating mitochondrial function and neuroinflammation. Prog Neuropsychopharmacol Biol Psychiatry 76**:** 169-178.

10. Anda RF, Croft JB, Felitti VJ, Nordenberg D, Giles WH, Williamson DF*, et al.* (1999). Adverse Childhood Experiences and Smoking During Adolescence and Adulthood. JAMA 282**:** 1652-1658.

11. Anderson EL, Heron J, Ben-Shlomo Y, Kuh D, Cooper R, Lawlor DA*, et al.* (2017). Adversity in Childhood and Measures of Aging in Midlife: Findings From a Cohort of British Women. Psychol Aging 32**:** 521-530.

12. Arnal J, El Amrani A, Chatellier G, Menard J, & Michel J (1993). Cardiac Weight in Hypertension Induced by Nitric Oxide Synthase Blockade. Hypertension 22**:** 380-387.

13. Barhoumi T, Kasal DA, Li MW, Shbat L, Laurant P, Neves MF*, et al.* (2011). T Regulatory Lymphocytes Prevent Angiotensin II–Induced Hypertension and Vascular Injury. Hypertension 57**:** 469-476.

14. Baumeister D, Akhtar R, Ciufolini S, Pariante CM, & Mondelli V (2016). Childhood trauma and adulthood inflammation: a meta-analysis of peripheral C-reactive protein, interleukin-6 and tumour necrosis factor-α. Mol Psychiatry 21**:** 642-649.

15. Bayraktutan U, Draper N, Lang D, & Shah AM (1998). Expression of functional neutrophil-type NADPH oxidase in cultured rat coronary microvascular endothelial cells. Cardiovasc Res 38**:** 256-262.

16. Bellavance M, A., & Rivest S (2014). The HPA-immune axis and the immunomodulatory actions of glucocorticoids in the brain. Front Immunol 5**:** 136.

17. Bellinger DL, MillE BA, Perez S, Carter J, Wood C, ThyagaRajan S*, et al.* (2008). Sympathetic modulation of immunity: relevance to disease. Cell Immunol 252 27-56.

18. Bellis MA, Hughes K, Leckenby N, Hardcastle C, & Lowey H (2014). Measuring mortality and the burden of adult disease associated with adverse childhood experiences in England: a national survey. J Public Health 37**:** 445-454.

19. Beltowski J (2006). Role of leptin in blood pressure regulation and arterial hypertension. J Hypertens 24.

20. Ben-Sasson SZ, Hu-Li J, Quiel J, Cauchetaux S, Ratner M, Shapira I*, et al.* (2009). IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation. Proc Natl Acad Sci U S A 106**:** 7119-7124.

21. Benjamin EJ, Blaha MJ, Chiuve SE, Cushman M, Das SR, Deo R*, et al.* (2017). Heart Disease and Stroke Statistics-2017 Update: A Report From the American Heart Association. Circulation 135**:** e146-e603.

22. Bianchi G, Fox U, Di Francesco GF, Giovanetti AM, & Pagetti D (1974). Blood pressure changes produced by kidney cross-transplantation between spontaneously hypertensive rats and normotensive rats. Clin Sci Mol Med 47**:** 435-448.

23. Bogdan (2015). Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol 36**:** 161-178.

24. Bogdan C (2001). Nitric oxide and the immune response. Nat Immunol 2**:** 907-916.

25. Bogdan C (2011). Regulation of lymphocytes by nitric oxide. Methods Mol Biol 677.

26. Bronte V, & Pittet MJ (2013). The spleen in local and systemic regulation of immunity. Immunity 39**:** 806-818.

27. Busillo JM, Azzam KM, & Cidlowski JA (2011). Glucocorticoids sensitize the innate immune system through regulation of the NLRP3 inflammasome. J Biol Chem 286**:** 38703-38713.

28. Camacho P, Fan H, Liu Z, & He JQ (2016). Small mammalian animal models of heart disease. Am J Cardiovasc Dis 6**:** 70-80.

29. Campbell F, Conti G, Heckman JJ, Moon SH, Pinto R, Pungello E*, et al.* (2014). EARLY CHILDHOOD INVESTMENTS SUBSTANTIALLY BOOST ADULT HEALTH. Science 343**:** 1478-1485.

30. Campbell JJ, Bowman EP, Murphy K, Youngman KR, Siani MA, Thompson DA*, et al.* (1998). 6-C-kine (SLC), a lymphocyte adhesion-triggering chemokine expressed by high endothelium, is an agonist for the MIP-3beta receptor CCR7. J Cell Biol 141**:** 1053-1059.

31. Carboni L, Becchi S, Piubelli C, Mallei A, Giambelli R, Razzoli M*, et al.* (2010). Early-life stress and antidepressants modulate peripheral biomarkers in a gene-environment rat model of depression. Prog Neuropsychopharmacol Biol Psychiatry 34**:** 1037-1048.

32. Chan CT, Moore JP, Budzyn K, Guida E, Diep H, Vinh A*, et al.* (2012). Reversal of Vascular Macrophage Accumulation and Hypertension by a CCR2 Antagonist in Deoxycorticosterone/ Salt-Treated Mice. Hypertension 60**:** 1207-1212.

33. Cho HJ, Bower JE, Kiefe CI, Seeman TE, & Irwin MR (2012). Early life stress and inflammatory mechanisms of fatigue in the Coronary Artery Risk Development in Young Adults (CARDIA) study. Brain Behav Immun 26**:** 859-865.

34. Coelho R, Viola TW, Walss-Bass C, Brietzke E, & Grassi-Oliveira R (2014). Childhood maltreatment and inflammatory markers: a systematic review. Acta Psychiatr Scand 129**:** 180-192.

35. Cornelius DC, Hogg JP, Scott J, Wallace K, Herse F, Moseley J*, et al.* (2013). Administration of interleukin-17 soluble receptor C suppresses TH17 cells, oxidative stress, and hypertension in response to placental ischemia during pregnancy. Hypertension 62**:** 1068-1073.

36. Crowell JA, Davis CR, Joung KE, Usher N, McCormick SP, Dearing E*, et al.* (2016). Metabolic pathways link childhood adversity to elevated blood pressure in midlife adults. Obes Res Clin Pract 10**:** 580-588.

37. Crowley SD, Song YS, Lin EE, Griffiths R, Kim HS, & Ruiz P (2010). Lymphocyte responses exacerbate angiotensin II-dependent hypertension. Am J Physiol Regul Integr Comp Physiol 298**:** R1089-1097.

38. Curtis JJ, Luke RG, Dustan HP, Kashgarian M, Whelchel JD, Jones P*, et al.* (1983). Remission of essential hypertension after renal transplantation. N Engl J Med 309**:** 1009-1015.

39. Dahl LK, & Heine M (1975). Primary role of renal homografts in setting chronic blood pressure levels in rats. Circ Res 36**:** 692-695.

40. Dahl LK, Heine M, & Thompson K (1974). Genetic influence of the kidneys on blood pressure-. Evidence from chronic renal homografts in rats with opposite predisposition to hypertension. Circ Res XXXIV**:** 94-101.

41. Dahly AJ, Hoagland KM, Flasch AK, Jha S, Ledbetter SR, & Roman RJ (2002). Antihypertensive effects of chronic anti-TGF-β antibody therapy in Dahl S rats. Am J Physiol Regul Integr Comp Physiol 283**:** R757-R767.

42. Danese A, & J Lewis S (2017). Psychoneuroimmunology of Early-Life Stress: The Hidden Wounds of Childhood Trauma? Neuropsychopharmacology 41**:** 99-114.

43. Danese A, & McEwen BS (2012). Adverse childhood experiences, allostasis, allostatic load, and age-related disease. Physiol Behav 106.

44. Danese A, Moffitt TE, Harrington H, Milne BJ, Polanczyk G, Pariante CM*, et al.* (2009). Adverse childhood experiences and adult risk factors for age-related disease: depression, inflammation, and clustering of metabolic risk markers. Arch Pediatr Adolesc Med 163**:** 1135-1143.

45. Danese A, Pariante CM, Caspi A, Taylor A, & Poulton R (2007). Childhood maltreatment predicts adult inflammation in a life-course study. Proc Natl Acad Sci U S A 104**:** 1319-1324.

46. Daniels WMU, Pietersen CY, Cartens ME, & Stein DJ (2004). Maternal Separation in Rats Leads to Anxiety-Like Behavior and a Blunted ACTH Response and Altered Neurotransmitter Levels in Response
to a Subsequent Stressor. Metab Brain Dis 19.

47. Darwiche SS, Pfeifer R, Menzel C, Ruan X, Hoffman M, Cai C*, et al.* (2012). Inducible nitric oxide synthase contributes to immune dysfunction following trauma. Shock 38**:** 499-507.

48. Davenport AP, Hyndman KA, Dhaun N, Southan C, Kohan DE, Pollock JS*, et al.* (2016). Endothelin. Pharmacol Rev 68**:** 357-418.

49. De Ciuceis C, Amiri F, Brassard P, Endemann DH, Touyz RM, & Schiffrin EL (2005). Reduced Vascular Remodeling, Endothelial Dysfunction, and Oxidative Stress in Resistance Arteries of Angiotensin II–Infused Macrophage Colony-Stimulating Factor–Deficient Mice. Arterioscler Thromb Vasc Biol 25**:** 2106-2113.

50. De Filippo K, Dudeck A, Hasenberg M, Nye E, Rooijen NV, Hartmann K*, et al.* (2013). Mast cell and macrophage chemokines CXCL1/CXCL2 control the early stage of neutrophil recruitment during tissue inflammation. Blood 121**:** 4930-4937.

51. de la Rosa M, Rutz S, Dorninger H, & Scheffold A (2004). Interleukin-2 is essential for CD4+CD25+ regulatory T cell function. Eur J Immunol 34**:** 2480-2488.

52. De Miguel C, Das S, Lund H, & Mattson DL (2010). T lymphocytes mediate hypertension and kidney damage in Dahl salt-sensitive rats. Am J Physiol Regul Integr Comp Physiol 298**:** R1136-1142.

53. De Miguel C, Lund H, & Mattson DL (2011). High dietary protein exacerbates hypertension and renal damage in Dahl SS rats by increasing infiltrating immune cells in the kidney. Hypertension 57**:** 269-274.

54. Denoeud J MM (2011). Role of CD27/CD70 pathway of activation in immunity and tolerance. J Leukoc Biol 2.

55. Deschenes SS, Graham E, Kivimaki M, & Schmitz N (2018). Adverse Childhood Experiences and the Risk of Diabetes: Examining the Roles of Depressive Symptoms and Cardiometabolic Dysregulations in the Whitehall II Cohort Study. Diabetes Care.

56. Didion SP, Kinzenbaw DA, Schrader LI, Chu Y, & Faraci FM (2009). Endogenous Interleukin-10 Inhibits Angiotensin II-Induced Vascular Dysfunction. Hypertension 54**:** 619-624.

57. Dinh QN, Drummond GR, Sobey CG, & Chrissobolis S (2014). Roles of inflammation, oxidative stress, and vascular dysfunction in hypertension. Biomed Res Int 2014**:** 406960.

58. Dong M, Giles WH, Felitti VJ, Dube SR, Williams JE, Chapman DP*, et al.* (2004). Insights Into Causal Pathways for Ischemic Heart Disease Adverse Childhood Experiences Study. Circulation 110**:** 1761-1766.

59. Dowling DJ, & Levy O (2014). Ontogeny of early life immunity. Trends Immunol 35**:** 299-310.

60. Duncan AE, Auslander WF, Bucholz KK, Hudson DL, Stein RI, & White NH (2015). Relationship Between Abuse and Neglect in Childhood and Diabetes in Adulthood: Differential Effects by Sex, National Longitudinal Study of Adolescent Health. Prev Chronic Dis 12**:** 1-14.

61. Ehrlich KB, Ross KM, Chen E, & Miller GE (2016). Testing the biological embedding hypothesis: Is early life adversity associated with a later proinflammatory phenotype? Dev Psychopathol 28**:** 1273-1283.

62. Eikelis N, Schlaich M, Aggarwal A, Kaye D, & Esler M (2003). Interactions Between Leptin and the Human Sympathetic Nervous System
. Hypertension 41**:** 1072-1079.

63. El Sabbahy M, & Vaidya VS (2011). Ischemic kidney injury and mechanism of tissue repair. Wiley Interdiscip Rev Syst Biol Med 3**:** 606-618.

64. Elenkov IJ (2008). Neurohormonal-cytokine interactions: implications for inflammation, common human diseases and well-being. Neurochem Int 52**:** 40-51.

65. Elisa T, Antonio P, Giuseppe P, Alessandro B, Giuseppe A, Federico C*, et al.* (2015). Endothelin Receptors Expressed by Immune Cells Are Involved in Modulation of Inflammation and in Fibrosis: Relevance to the Pathogenesis of Systemic Sclerosis. J Immunol Res 2015**:** 11.

66. Elliot AJ, & Chapman BP (2016). Socioeconomic status, psychological resources, and inflammatory markers: Results from the MIDUS Study. Health Psychol 35**:** 1205-1213.

67. Elmarakby AA, Quigley JE, Imig JD, Pollock JS, & Pollock DM (2008). TNF-alpha inhibition reduces renal injury in DOCA-salt hypertensive rats. Am J Physiol Regul Integr Comp Physiol 294**:** R76-83.

68. Elmarakby AA, Quigley JE, Olearczyk JJ, Sridhar A, Cook AK, Inscho EW*, et al.* (2007). Chemokine Receptor CCR2b Inhibition Provides Renal Protection in Angiotensin II-Salt Hypertension. Hypertension 50**:** 1069-1076.

69. Elmarakby AA, Quigley JE, Pollock DM, & Imig JD (2006). Tumor necrosis factor alpha blockade increases renal Cyp2c23 expression and slows the progression of renal damage in salt-sensitive hypertension. Hypertension 47**:** 557-562.

70. Elwenspoek MMC, Hengesch X, Leenen FAD, Schritz A, Sias K, Schaan VK*, et al.* (2017). Proinflammatory T Cell Status Associated with Early Life Adversity. J Immunol 199.

71. Felitti VJ, Anda RF, Nordenberg D, Williamson DF, Spitz AM, Edwards V*, et al.* (1998). Relationship of Childhood Abuse and Household Dysfunction to Many of the

Leading Causes of Death in Adults. The adverse childhood experiences study. Am J Prev Med 14**:** 245-258.

72. Flierl MA, Rittirsch D, Huber-Lang M, Sarma JV, & Ward PA (2008). Catecholamines—Crafty Weapons in the Inflammatory Arsenal of Immune/Inflammatory Cells or Opening Pandora's Box§? Mol Med 14**:** 195-204.

73. Florquin S, Amraoui Z, Dubois C, Decuyper J, & Goldman M (1994). The Protective Role of Endogenously Synthesized Nitric Oxide in Staphylococcal Enterotoxin B-induced Shock in Mice. J Exp Med 180**:** 1153-1158.

74. Forster R, Schubel A, Breitfeld D, Kremmer E, Renner-Muller I, Wolf E*, et al.* (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. Cell 99**:** 22-33.

75. Forstermann U, & Kleinert H (1995). Nitric oxide synthase: expression and expressional control of the three isoforms. Naunyn Schmiedebergs Arch Pharmacol 352**:** 351-364.

76. Forstermann U, & Sessa WC (2012). Nitric oxide synthases: regulation and function. Eur Heart J 33**:** 829-837.

77. Francis DD, Caldji C, Champagne F, Plotsky PM, & Meaney MJ (1999). The role of corticotropin-releasing factor--norepinephrine systems in mediating the effects of early experience on the development of behavioral and endocrine responses to stress. Biol Psychiatry 46**:** 1153-1166.

78. Franco JG, Lisboa PC, Lima NS, Amaral TA, Peixoto-Silva N, Resende AC*, et al.* (2013). Resveratrol attenuates oxidative stress and prevents steatosis and hypertension in obese rats programmed by early weaning. J Nutr Biochem 24**:** 960-966.

79. Franco M, Martinez F, Quiroz Y, Galicia O, Bautista R, Johnson RJ*, et al.* (2007). Renal angiotensin II concentration and interstitial infiltration of immune cells are correlated with blood pressure levels in salt-sensitive hypertension. Am J Physiol Regul Integr Comp Physiol 293**:** R251-256.

80. Fuller-Thomson E, Roane JL, & Brennenstuhl S (2016). Three Types of Adverse Childhood Experiences, and Alcohol and Drug Dependence Among Adults: An Investigation Using Population-Based Data. Subst Use Misuse 51**:** 1451-1461.

81. Furuichi K, Gao JL, Horuk R, Wada T, Kaneko S, & Murphy PM (2008). Chemokine Receptor CCR1 Regulates Inflammatory Cell Infiltration after Renal Ischemia-Reperfusion Injury. J Immunol 181**:** 8670-8676.

82. Garcia AG, Wilson RM, Heo J, Murthy NR, Baid S, Ouchi N*, et al.* (2012). Interferon-γ ablation exacerbates myocardial hypertrophy in diastolic heart failure. Am J Physiol Heart Circ Physiol 303**:** H587-H596.

83. Gardiner SM, Kemp PA, Bennett T, Palmer RM, & Moncada S (1992). Nitric oxide synthase inhibitors cause sustained, but reversible, hypertension and hindquarters vasoconstriction in Brattleboro rats. Eur J Pharmacol 213**:** 449-451.

84. Genest SE, Gulemetova R, Laforest S, Drolet G, & Kinkead R (2004). Neonatal maternal separation and sex-specific plasticity of the hypoxic ventilatory response in awake rat. J Physiol 554**:** 543-557.

85. George ED, Bordner KA, Elwafi HM, & Simen AA (2010). Maternal separation with early weaning: a novel mouse model of early life neglect. BMC Neurosci 11**:** 1-14.

86. Gilbert LK, Breiding MJ, Merrick MT, Thompson WW, Ford DC, Dhingra SS*, et al.* (2015). Childhood Adversity and Adult Chronic Disease. Am J Prev Med 48**:** 345-349.

87. Gonzalez-Amaro R, Cortes JR, Sanchez-Madrid F, & Martin P (2013). Is CD69 an effective brake to control inflammatory diseases? Trends Mol Med 19**:** 625-632.

88. Goodwin RD, & Stein MB (2004). Association between childhood trauma and physical disorders among adults in the United States. Psychol Med 34**:** 509-520.

89. Goral S (2011). The three-signal hypothesis of lymphocyte activation/targets for immunosuppression. Dial Transplant 40**:** 14-16.

90. Gorman AM, Heavey B, Creagh E, Cotter TG, & Samali A (1999). Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. FEBS Lett 445**:** 98-102.

91. Griebel G, Belzung C, Perrault G, & Sanger DJ (2000). Differences in anxiety-related behaviours and in sensitivity to diazepam in inbred and outbred strains of mice. Psychopharmacology 148**:** 164-170.

92. Griendling KK, Minieri CA, Ollerenshaw JD, & Alexander RW (1994). Angiotensin II stimulates NADH and NADPH oxidase activity in cultured vascular smooth muscle cells. Circ Res 74**:** 1141-1148.

93. Griffith JW, Sokol CL, & Luster AD (2014). Chemokines and chemokine receptors: positioning cells for host defense and immunity. Annu Rev Immunol 32**:** 659-702.

94. Grishman EK, White PC, & Savani RC (2012). Toll-like receptors, the NLRP3 inflammasome, and interleukin-1b in the development and progression of type 1 diabetes. Pediatr Res 71**:** 626-632.

95. Groom JR, & Luster AD (2011). CXCR3 in T cell function. Exp Cell Res 317**:** 620-631.

96. Guo J, Guan Q, Liu X, Wang H, Gleave ME, Nguan CY*, et al.* (2016). Relationship of clusterin with renal inflammation and fibrosis after the recovery phase of ischemia-reperfusion injury. BMC Nephrol 17.

97. Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S*, et al.* (2007). Role of the T cell in the genesis of angiotensin II induced hypertension and vascular dysfunction. J Exp Med 204**:** 2449-2460.

98. Harmon A, Cornelius D, Amaral L, Paige A, Herse F, Ibrahim T*, et al.* (2015). IL-10 supplementation increases Tregs and decreases hypertension in the RUPP rat model of preeclampsia. Hypertens Pregnancy 34**:** 291-306.

99. Hautefort A, Girerd B, Montani D, Cohen-Kamisty S, Price L, Lambrecht BN*, et al.* (2015). T-Helper 17 Cell Polarization in Pulmonary Arterial Hypertension. Chest 147**:** 1610-1620.

100. Herberth G, Weber A, Ro¨der S, Elvers HD, Kra¨mer U, Schins RPF*, et al.* (2008). Relation between stressful life events, neuropeptides and cytokines: results from the LISA birth cohort study. Pediatr Allergy Immunol 19**:** 722-729.

101. Hermann M, Flammer A, & Luscher TF (2006). Nitric Oxide in Hypertension. The Journal of Clinical Hypertension 8**:** 17-29.

102. Ho DH, Burch ML, Musall B, Musall JB, Hyndman KA, & Pollock JS (2016). Early life stress in male mice induces superoxide production and endothelial dysfunction in adulthood. Am J Physiol Heart Circ Physiol 310**:** H1267-1274.

103. Hoch NE, Guzik TJ, Chen W, Deans T, Maalouf SA, Gratze P*, et al.* (2009). Regulation of T-cell function by endogenously produced angiotensin II. Am J Physiol Regul Integr Comp Physiol 296**:** R208-216.

104. Hocher B (2014). More than genes: the advanced fetal programming hypothesis. J Reprod Immunol 104-105**:** 8-11.

105. Hohmann CF, Odebode G, Naidu L, & Koban M (2017). Early Life Stress Alters Adult Inflammatory Responses in a Mouse Model for Depression. Ann Psychiatry Ment Health 5**:** 1095.

106. Hong S, Nelesen RA, Krohn PL, Mills PJ, & Dimsdale JE (2006). The association of social status and blood pressure with markers of vascular inflammation. Psychosom Med 68**:** 517-523.

107. Hostinar CE, Lachman ME, Mroczek DK, Seeman TE, & Miller GE (2015). Additive contributions of childhood adversity and recent stressors to inflammation at midlife: Findings from the MIDUS study. Dev Psychol 51**:** 1630-1644.

108. Howe LD, Galobardes B, Sattar N, Hingorani AD, Deanfield J, Ness AR*, et al.* (2010). Are there socioeconomic inequalities in cardiovascular risk factors in childhood, and are they mediated by adiposity? Findings from a prospective cohort study. Int J Obes 24**:** 1149-1159.

109. Huang B, Cheng Y, Usa K, Liu Y, Baker MA, Mattson DL*, et al.* (2016). Renal Tumor Necrosis Factor α Contributes to Hypertension in Dahl Salt-Sensitive Rats. Sci Rep 2**:** 21960.

110. Huang JL, Zhang YL, Wang CC, Zhou JR, Ma Q, Wang X*, et al.* (2012). Enhanced phosphorylation of MAPKs by NE promotes TNF-α production by macrophage through α adrenergic receptor. Inflammation 35**:** 527-534.

111. Huang L, Wang A, Hao Y, Li W, Liu C, Yang Z*, et al.* (2018). Macrophage Depletion Lowered Blood Pressure and Attenuated Hypertensive Renal Injury and Fibrosis. Front Physiol 9**:** 473.

112. Huang TJ, Macary PA, Kemeny DM, & Chung KF (1999). Effect of CD8+ T-cell depletion on bronchial hyper-responsiveness and inflammation in sensitized and allergen-exposed Brown–Norway rats. Immunology 96**:** 416-423.

113. Huffhines L, Noser A, & Patton SR (2016). The Link Between Adverse Childhood Experiences and Diabetes. Curr Diab Rep 16**:** 54.

114. Hyndman KA, MacDonell AH, & Pollock JS (2012). Extracellular signal-regulated kinases 1/2 signaling pathways are not involved in endothelin regulation of mouse inner medullary collecting duct nitric oxide production. Life Sci 91**:** 578-582.

115. Hyndman KA, & Pollock JS (2014). NO and the A and B of Endothelin of Sodium Homeostasis. Curr Opin Nephrol Hypertens 22**:** 26-31.

116. Ibiza S, & Serrador JM (2008). The role of nitric oxide in the regulation of adaptive immune responses. Immunologia 27**:** 103-117.

117. Imakiire T, Kikuchi Y, Yamada M, Kushiyama T, Higashi K, Hyodo N*, et al.* (2007). Effects of renin-angiotensin system blockade on macrophage infiltration in patients with hypertensive nephrosclerosis. Hypertens Res 30**:** 635-642.

118. Ishibashi M, Hiasa K, Zhao Q, Inoue S, Ohtani K, Kitamoto S*, et al.* (2004). Critical role of monocyte chemoattractant protein-1 receptor CCR2 on monocytes in hypertension-induced vascular inflammation and remodeling. Circ Res 94**:** 1203-1210.

119. Ishimitsu T, Uehara Y, Numabe A, Tsukada H, Ogawa Y, & Yagi S (1993). Antihypertensive effect of interleukin-2 in salt-sensitive Dahl rats. . Hypertension 23**:** 68-73.

120. Isohookana R, Marttunen M, Hakko H, Riipinen P, & Riala K (2016). The impact of adverse childhood experiences on obesity and unhealthy weight control behaviors among adolescents. Compr Psychiatry 71**:** 17-24.

121. Itani HA, Xiao L, Saleh MA, Wu J, Pilkinton MA, Dale BL*, et al.* (2016). CD70 Exacerbates Blood Pressure Elevation and Renal Damage in Response to Repeated Hypertensive Stimuli. Circ Res 118**:** 1233-1243.

122. Jordan AR, Racine RR, Henning MJ, & Lokeshwar VB (2015). The Role of CD44 in Disease Pathophysiology and Targeted Treatment. Front Immunol 6.

123. Jovic S, Linge HM, Shikhagaie MM, Olin AL, Lannefors L, Erjefa ̈lt JS*, et al.* (2016). The neutrophil-recruiting chemokine GCP-2/CXCL6 is expressed in cystic fibrosis airways and retains its functional properties after binding to extracellular DNA. Mucosal Immunol 9**:** 112-123.

124. Jun Z, Hill PA, Lan HY, Foti R, Mu W, Atkins RC*, et al.* (1997). CD44 and Hyaluronan expression in the development of experimental crescentic glomerulonephritis. Clin Exp Immunol 108**:** 69-77.

125. Kahler J, Ewert A, Weckmuller J, Stobbe S, Mittmann C, Koster R*, et al.* (2001). Oxidative stress increases endothelin-1 synthesis in human coronary artery smooth muscle cells. J Cardiovasc Pharmacol 38**:** 49-57.

126. Kamat NV, Thabet SR, Xiao L, Saleh MA, Kirabo A, Madhur MS*, et al.* (2015). Renal transporter activation during angiotensin-II hypertension is blunted in interferon-gamma-/- and interleukin-17A-/- mice. Hypertension 65**:** 569-576.

127. Kawabe K, Watanbe TX, Kumiko S, & Sokabe H (1978). Influence on blood pressure of renal isografts between spontaneously hypertensive and normotensive rats, utilizing the F1 hybrids. Jpn Heart J 20**:** 886-894.

128. Kazumi T, Kawaguchi A, Katoh J, Iwahashi M, & Yoshino G (1999). Fasting insulin and leptin serum levels are associated with systolic blood pressure independent of percentage body fat and body mass index. J Hypertens 17**:** 1451-1455.

129. Keller AM, Schildknecht A, Xiao Y, Van den Broek M, & Borst J (2008). Expression of costimulatory ligand CD70 on steady-state dendritic cells breaks CD8+ T cell tolerance and permits effective immunity. Immunity 29**:** 934-946.

130. Khera R, Lu Y, Lu J, Saxena A, Nasir K, Jiang L*, et al.* (2018). Impact of 2017 ACC/AHA guidelines on prevalence of hypertension and eligibility for antihypertensive

treatment in United States and China: nationally representative cross sectional study. BMJ 362**:** k2357.

131. Kiecolt-Glaser JK, Gouin JP, Weng N, Malarkey WB, Beversdorf DQ, & Glaser R (2011). Childhood Adversity Heightens the Impact of Later-Life Caregiving Stress on Telomere Length and Inflammation. Psychosom Med 73**:** 16-22.

132. Kim HY, & H.S. K (2014). IL-10 up-regulates CCL5 expression in vascular smooth muscle cells from spontaneously hypertensive rats. Cytokine 68**:** 40-49.

133. Kirabo A, Fontana V, de Faria AP, Loperena R, Galindo CL, Wu J*, et al.* (2014). DC isoketal-modified proteins activate T cells and promote hypertension. J Clin Invest 124**:** 4642-4656.

134. Kohan DE, & Barton M (2014). Endothelin and endothelin antagonists in chronic kidney disease. Kidney Int 86**:** 896-904.

135. Kol A, Bourcier T, Lichtman AH, & Libby P (1999). Chlamydial and human heat shock protein 60s activate human vascular endothelium, smooth muscle cells, and macrophages. J Clin Invest 103**:** 571-577.

136. Koscielsk-Kasprzak K, Bartoszek D, Myszka M, Zabinska M, & Klinger M (2014). The complement cascade and renal disease. Arch Immunol Ther Exp 62**:** 47-57.

137. Kossmann S, Schwenk M, Hausding M, Karbach SH, Schmidgen MI, Brandt M*, et al.* (2013). Angiotensin II-induced vascular dysfunction depends on interferon-γ-driven immune cell recruitment and mutual activation of monocytes and NK-cells. Arterioscler Thromb Vasc Biol 33**:** 1313-1319.

138. Kuras YI, McInnis CM, Thoma MV, Chen X, Hanlin L, Gianferante D*, et al.* (2017). Increased alpha-amylase response to an acute psychosocial stress challenge in healthy adults with childhood adversity. Dev Psychobiol 59**:** 91-98.

139. Ladd CO, Huot RL, Thrivikraman KV, Nemeroff CB, Michael J. Meaney MJ, & Plotsky PM (2000). Long-term behavioral and neuroendocrine adaptations to adverse early experience. Prog Brain Res 122**:** 81-103.

140. Landmesser U, Cai H, Dikalov S, McCann L, Hwang J, Jo H*, et al.* (2002). Role of p47(phox) in vascular oxidative stress and hypertension caused by angiotensin II. Hypertension 40**:** 511-515.

141. Landmesser U, Dikalov S, Price SR, McCann LA, Fukai T, Holland SM*, et al.* (2003). Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. J Clin Invest 111**:** 1201-1209.

142. Lanzavecchia A, & Sallusto F (2000). Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science 290**:** 92-97.

143. Larbi A, & Fulop T (2014). From "truly naive" to "exhausted senescent" T cells: when markers predict functionality. Cytometry A 85**:** 25-35.

144. Lee C, Tsenkova V, & Carr D (2014). Childhood trauma and metabolic syndrome in men and women. Soc Sci Med 105**:** 122-130.

145. Lee DL, Sturgis LC, Labazi H, Osborne JB, Fleming C, Pollock JS*, et al.* (2006). Angiotensin II hypertension is attenuated in interleukin-6 knockout mice. Am J Physiol Herat Circ Physiol 290**:** H935-H940.

146. Lemieux A, Coe CL, & Carnes M (2008). Symptom Severity Predicts Degree of T Cell Activation In Adult Women Following Childhood Maltreatment. Brain Behav Immun 22**:** 994-1003.

147. Levine S (1967). Maternal and Environmental Influences on the Adrenocortical Response to Stress in
Weanling Rats. Science 156**:** 258-260.

148. Li A, Tu MT, Sousa AC, Alvarado B, Kone GK, Guralnik J*, et al.* (2015). Early life adversity and C-reactive protein in diverse populations of older adults: a cross-sectional analysis from the International Mobility in Aging Study (IMIAS). BMC Geriatr 12**:** 102.

149. Lima VV, Zemse SM, Chiao C, Bomfim GF, Tostes RC, Webb RC*, et al.* (2016). Interleukin-10 limits increased blood pressure and vascular RhoA/Rho-kinase signaling in angiotensin II-infused mice. Life Sci 145**:** 137-143.

150. Lippmann M, Bress A, Nemeroff CB, Plotsky PM, & Monteggia LM (2007). Long-term behavioural and molecular alterations associated with maternal separation in rats. Eur J Neurosci 25**:** 3091-3098.

151. Liu D, Caldji C, Sharma S, Plotsky PM, & Meaney MJ (2000). Influence of neonatal rearing conditions on stress-induced adrenocorticotropin responses and norepinepherine release in the hypothalamic paraventricular nucleus. J Neuroendocrinol 12**:** 5-12.

152. Liu D, Diorio J, Tannenbaum B, Caldji C, Francis D, Freedman A*, et al.* (1977). Maternal care, hippocampal glucocorticoid receptors and hypothalamic-pituitary-adrenal responses to stress. Science 277**:** 1659-1662.

153. Liu J, Yang F, Yang XP, Jankowski M, & Pagano PJ (2003). NAD(P)H oxidase mediates angiotensin II-induced vascular macrophage infiltration and medial hypertrophy. Arterioscler Thromb Vasc Biol 23**:** 776-782.

154. Liu Y, Rafferty TM, Rhee SW, Webber JS, Song L, Ko B*, et al.* (2017). CD8+ T cells stimulate Na-Cl co-transporter NCC in distal convoluted tubules leading to salt-sensitive hypertension. Nature Communications 8**:** 14037.

155. Liu YZ, Wang YX, & Jiang CL (2017). Inflammation: The Common Pathway of Stress-Related Diseases. Front Hum Neurosci 11**:** 360.

156. Loria AS, Brands MW, Pollock DM, & Pollock JS (2013). Early life stress sensitizes the renal and systemic sympathetic system in rats. Am J Physiol Renal Physiol 305**:** F390-F395.

157. Loria AS, D'Angelo G, Pollock DM, & Pollock JS (2010). Early life stress downregulates endothelin receptor expression and enhances acute stress-mediated blood pressure responses in adult rats. Am J Physiol Regul Integr Comp Physiol 299**:** R185-191.

158. Loria AS, Kang KT, Pollock DM, & Pollock JS (2011). Early life stress enhances angiotensin II-mediated vasoconstriction by reduced endothelial nitric oxide buffering capacity. Hypertension 58**:** 619-626.

159. Loria AS, & Osborn JL (2017). Maternal separation diminishes -adrenergic receptor density and function in renal vasculature from male Wistar-Kyoto rats. Am J Physiol Renal Physiol 313**:** F47-F54.

160. Loria AS, Pollock DM, & Pollock JS (2010). Early life stress sensitizes rats to angiotensin II-induced hypertension and vascular inflammation in adult life. Hypertension 55**:** 494-499.

161. Loria AS, Yamamoto T, Pollock DM, & Pollock JS (2013). Early life stress induces renal dysfunction in adult male rats but not female rats. Am J Physiol Regul Integr Comp Physiol 304**:** R121-129.

162. MacMicking J, Xie QW, & Nathan C (1997). Nitric oxide and macrophage function. Annu Rev Immunol 15**:** 323-350.

163. Madhur MS, Lob HE, McCann LA, Iwakura Y, Blinder Y, Guzik TJ*, et al.* (2010). INTERLEUKIN 17 PROMOTES ANGIOTENSIN II-INDUCED HYPERTENSION AND VASCULAR DYSFUNCTION. Hypertension 55.

164. Maes M, Van der Planken M, Stevens WJ, Peeters D, DeClerck LS, Bridts CH*, et al.* (1992). Leukocytosis, monocytosis and neutrophilia: hallmarks of severe depression. J Psychiatr Res 26**:** 125-134.

165. Manning AM, Bell FP, Rosenbloom CL, Chosay JG, Simmons CA, Northrup JL*, et al.* (1995). NF-kappa B is activated during acute inflammation in vivo in association with elevated endothelial cell adhesion molecule gene expression and leukocyte recruitment. J Inflamm 45**:** 283-296.

166. Mantovani A (1999). The Chemokine System: Redundancy for Robust Outputs. Immunol Today 20.

167. Marko L, Kvakan H, Park J, Qadri F, Spallek B, Binger KJ*, et al.* (2012). Interferon-γ Signaling Inhibition Ameliorates Angiotensin II–Induced Cardiac Damage. Hypertension 60**:** 1430-1436.

168. Martinelli R, Gegg M, Longbottom R, Adamson P, Turowski P, & Greenwood J (2009). ICAM-1-mediated endothelial nitric oxide synthase activation via calcium and AMP-activated protein kinase is required for transendothelial lymphocyte migration. Mol Biol Cell 20**:** 995-1005.

169. Mastorakos G, Pavlatou MG, & Mizamtsidi M (2006). The Hypothalamic-Pituitary-Adrenal and the Hypothalamic Pituitary-Gonadal Axes Interplay. Pediatr Endocrinol Rev 3**:** 172-181.

170. Matsuno K, Yamada H, Iwata K, Jin D, Katsuyama M, Matsuki M*, et al.* (2005). Nox1 is involved in angiotensin II-mediated hypertension: a study in Nox1-deficient mice. Circulation 112**:** 2677-2685.

171. Mattson DL, Lund H, Guo C, Rudemiller N, Geurts AM, & Jacob H (2013). Genetic mutation of recombination activating gene 1 in Dahl salt-sensitive rats attenuates hypertension and renal damage. Am J Physiol Regul Integr Comp Physiol 304**:** R407-R414.

172. McAuley JD, Stewart AL, Webber ES, Cromwell HC, Servatius RJ, & Pang KCH (2009). Wistar-Kyoto rats as an animal model of anxiety vulnerability: Support for a hypervigilance hypothesis. Behav Brain Res 204**:** 162-168.

173. McCall TB, Boughton-Smith NK, Palmer RM, Whittle BJ, & Moncada S (1989). Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. Biochem J 261**:** 293-296.

174. McEwen BS (2003). Early life influences on life-long patterns of behavior and health. Ment Retard Dev Disabil Res Rev 9**:** 149-154.

175. McKay DM, Lu J, Jedrzkiewicz S, Ho W, & Sharkey KA (1999). Nitric Oxide Participates in the Recovery of Normal Jejunal Epithelial Ion Transport Following Exposure to the Superantigen, Staphylococcus aureus Enterotoxin B. J Immunol 163**:** 4519-4526.

176. Medzhitov R (2001). Toll-like receptors and innate immunity. Nat Rev Immunol 1**:** 135-145.

177. Merai R, Siegal C, Rakotz M, Basch P, Wright J, Wong B*, et al.* (2016). Centers for Disease Control and Preventio Ground Rounds: A public Health Approach to Detect and Control Hypertension. Morb Mortal Weekly Rep 65**:** 1261-1264.

178. Mian MOR, Barhoumi T, Briet M, Paradis P, & Schiffrin EL (2016). Deficiency of T-regulatory cells exaggerates angiotensin II-induced microvascular injury by enhancing immune responses. Jornal of Hypertension 34**:** 97-108.

179. Milani-Nejad N, & Janssen PM (2014). Small and large animal models in cardiac contraction research: advantages and disadvantages. Pharmacol Ther 141**:** 235-249.

180. Miller GE, Chen E, Fok AK, Walker H, Lim A, Nicholls EF*, et al.* (2009). Low early-life social class leaves a biological residue manifested by decreased glucocorticoid and increased proinflammatory signaling. Proc Natl Acad Sci U S A 106**:** 14716-14721.

181. Mittal M, Siddiqui MR, Tran K, Reddy SP, & Malik AB (2014). Reactive Oxygen Species in Inflammation and Tissue Injury. Antioxid Redox Signal 20**:** 1126-1167.

182. Miura M, Fu X, Zhang Q, Remick DG, & Fairchild RL (2001). Neutralization of Gro-alpha and Macrophage Inflammatory Protein-2 Attenuates Renal Ischemia/Reperfusion Injury. Am J Pathol 159**:** 2137-2145.

183. Moes AD, Severs D, Verdonk K, van der Lubbe N, Zietse R, Danser AHJ*, et al.* (2018). Mycophenolate Mofetil Attenuates DOCA-Salt Hypertension: Effects on Vascular Tone. Front Physiol 9**:** 578.

184. Mohazzab KM, Kaminski PM, & Wolin MS (1994). NADH oxidoreductase is a major source of superoxide anion in bovine coronary artery endothelium. Am J Physiol 266**:** H2568-H2572.

185. Moncada S, Palmer RM, & Higgs EA (1989). Biosynthesis of nitric oxide from L-arginine. A pathway for the regulation of cell function and communication. Biochem Pharmacol 38**:** 1709-1715.

186. Moya-Perez A, Perez-Villalba A, Benitez-Paez A, Campillo I, & Sanz Y (2017). Bifidobacterium CECT 7765 modulates early stress-induced immune, neuroendocrine and behavioral alterations in mice. Brain Behav Immun 65**:** 43-56.

187. Mueller CF, Laude K, McNally JS, & Harrison DG (2005). ATVB in focus: redox mechanisms in blood vessels. Arterioscler Thromb Vasc, Biol 25**:** 274-278.

188. Murphy MO, Herald JB, Leachman J, Villasante Tezanos A, Cohn DM, & Loria AS (2018). A model of neglect during postnatal life heightens obesity-induced hypertension and is linked to a greater metabolic compromise in female mice. Int J Obes (Lond) 42**:** 1354-1365.

189. Murphy SR, Dahly-Vernon AJ, Dunn KMJ, Chen CCA, Ledbetter SR, Williams JM*, et al.* (2012). Renoprotective effects of anti-TGF-β antibody and antihypertensive therapies in Dahl S rats. Am J Physiol Regul Integr Comp Physiol 303**:** R57-R69.

190. Nakamura K, Hayashi H, & Kubokawa M (2015). Proinflammatory Cytokines and Potassium Channels in the Kidney. Mediators Inflamm 2015**:** 362768.

191. Nam H, Clinton SM, Jackson NL, & Kerman IA (2014). Learned helplessness and social avoidance in the Wistar-Kyoto rat. Front Behav Neurosci 8**:** 109.

192. Nguyen H, Chiasson VL, Chatterjee P, Kopriva SE, Young KJ, & Mitchell BM (2013). Interleukin-17 causes Rho-kinase-mediated endothelial dysfunction and hypertension. Cardiovasc Res 97**:** 696-704.

193. Niedbala W, Cai B, & Liew FY (2006). Role of nitric oxide in the regulation of T cell functions. Ann Rheum Dis 65**:** iii37-iii40.

194. Oh J, Wunsch R, Turzer M, Bahner M, Raggi P, Querfeld U*, et al.* (2002). Advanced coronary and carotid arteriopathy in young adults with childhood-onset chronic renal failure. Circulation 106**:** 100-105.

195. Oyola MG, & Handa RJ (2017). Hypothalamic–pituitary–adrenal and hypothalamic–pituitary– gonadal axes: sex differences in regulation of stress responsivity. Stress 20**:** 476-494.

196. Pace TWW, Mletxko TC, Alagbe O, Musselman DL, Nemeroff CB, Miller AH*, et al.* (2006). Increased Stress-Induced Inflammatory Responses in Male Patients With Major Depression and Increased Early Life Stress. Am J Psychiatry 163**:** 1630-1633.

197. Pagano PJ, Chanock SJ, Siwik DA, Colucci WS, & Clark JK (1998). Angiotensin II induces p67phox mRNA expression and NADPH oxidase superoxide generation in rabbit aortic adventitial fibroblasts. Hypertension 32**:** 331-337.

198. Pagano PJ, Ito Y, Tornheim K, Gallop PM, Tauber AI, & Cohen RA (1995). An NADPH oxidase superoxide-generating system in the rabbit aorta. Am J Physiol 268**:** H2274-H2280.

199. Papayannopoulos V, Metzler KD, Hakkim A, & Zychlinsky A (2010). Neutrophil elastase and myeloperoxidase regulate the formation of neutrophil extracellular traps. J Cell Biol 191**:** 677-691.

200. Patel R, Cardneau JD, Colles SM, & Graham LM (2006). Synthetic smooth muscle cell phenotype is associated with increased nicotinamide adenine dinucleotide phosphate oxidase activity: effect on collagen secretion. J Vasc Surg 43**:** 364-371.

201. Patouraux S, Rousseau D, Bonnafous S, Lebeaupin C, Luci C, Canivet CM*, et al.* (2017). CD44 is a key player in non-alcoholic steatohepatitis. J Hepatol.

202. Pfeffer CR, Altemus M, Heo M, & Jiang H (2007). Salivary Cortisol and Psychopathology in Children Bereaved by the September 11, 2001 Terror Attacks. Biol Psychiatry 61**:** 957-965.

203. Phillips JE, Marsland AL, Flory JD, Muldoon MF, Cohen S, & Manuck SB (2009). Parental Education is Related to C-Reactive Protein among Female Middle Aged Community Volunteers. Brain Behav Immun 23**:** 677-683.

204. Pinheiro ML, Ferraz-de-Paula V, Ribeiro A, Sakai M, Bernardi MM, & Palermo-Neto J (2011). Long-term maternal separation differentially alters serum corticosterone levels and blood neutrophil activity in A/J and C57BL/6 mouse offspring. Neuroimmunomodulation 18**:** 184-190.

205. Plotsky PM, Meaney MJ (1993). Early, postnatal experience alters hypothalamic corticotropin-releasing factor (CRF) mRNA, median eminence CRF content and stress-induced release in adult rats. Mol Brain Res 18.

206. Polla BS, & Cossarizza A (1996). Stress proteins in inflammation. EXS 77**:** 375-391.

207. Ponta H, Sherman L, & Herrlich PA (2003). CD44: from adhesion molecules to signalling regulators. Mat Rev Mol Cell Biol 4**:** 33-45.

208. Powell ND, Sloan EK, Bailey MT, Arevalo JM, Miller GE, Chen E*, et al.* (2013). Social stress up-regulates inflammatory gene expression in the leucocyte transcriptome via B-adrenergic induction of myelopoiesis. Proc Natl Acad Sci USA 110**:** 16574-16579.

209. Pu Q, Neves MF, Virdis A, Touyz RM, & Schiffrin EL (2003). Endothelin antagonism on aldosterone-induced oxidative stress and vascular remodeling. Hypertension 42**:** 49-55.

210. Rajagopalan S, Kurz S, Munzel T, Tarpey M, Freeman BA, Griendling KK*, et al.* (1996). Angiotensin II-mediated hypertension in the rat increases vascular superoxide production via membrane NADH/NADPH oxidase activation. Contribution to alterations of vasomotor tone. J Clin Invest 97**:** 1916-1923.

211. Rana S, Pugh PC, Jackson N, Clinton SM, & Kerman IA (2015). Inborn Stress Reactivity Shapes Adult Behavioral Consequences of Early-Life Maternal Separation Stress. Neurosci Lett 584**:** 146-150.

212. Recchia FA, & Lionetti V (2007). Animal models of dilated cardiomyopathy for translational research. Vet Res Commun 31 Suppl 1**:** 35-41.

213. Reho JJ, & Fisher SA (2015). The stress of maternal separation causes misprogramming in the postnatal maturation of rat resistance arteries. Am J Physiol Heart Circ Physiol 309**:** H1468-H1478.

214. Riba A, Olier M, Lacroix-Lamande S, Lencina C, Bacquie V, Harkat C*, et al.* (2018). Early life stress in mice is a suitable model for Irritable Bowel Syndrome but does not predispose to colitis nor increase susceptibility to enteric infections. Brain Behav Immun 73**:** 403-415.

215. Ribeiro MO, Antunes E, de Nucci G, Lovisolo SM, & Zatz R (1992). Chronic inhibition of nitric oxide synthesis. A new model of arterial hypertension. Hypertension 20**:** 298-303.

216. Rich-Edwards JW, Mason S, Rexrode K, Spiegelman D, Hibert E, Kawachi I*, et al.* (2012). Physical and sexual abuse in childhood as predictors of early onset cardiovascular events in women. Cuirculation 126**:** 920-927.

217. Rich-Edwards JW, Spiegelman D, Hibert ENL, Jun H, Todd TJ, Kawachi I*, et al.* (2010). Abuse in Childhood and Adolescence as a Predictor of Type 2 Diabetes in Adult Women. Am J Prev Med 39**:** 529-536.

218. Richard KS, Phoon A, Kitching R, Odobasic D, Jones LK, Semple TJ*, et al.* (2008). T-bet Deficiency Attenuates Renal Injury in Experimental Crescentic Glomerulonephritis. J Am Soc Nephrol 19**:** 477-485.

219. Riley EH, Wright RJ, Jun HJ, Hibert EN, & Rich-Edwards JW (2010). Hypertension in Adult Survivors of Child Abuse: Observations from the Nurses' Health Study II. J Epidemiol Community Health 64**:** 413-418.

220. Rodriguez-Iturbe B, Pons H, & Johnson RJ (2017). Role of the Immune System in Hypertension. Physiol Rev 97**:** 1127-1164.

221. Romeo RD, Bellani R, Karatsoreos IN, Chhua N, Vernov M, Conrad CD*, et al.* (2006). Stress History and Pubertal Development Interact to Shape Hypothalamic-Pituitary-Adrenal Axis Plasticity Endocrinology 147**:** 1664-1674.

222. Roque A, Ochoa-Zarzosa A, & Torner L (2016). Maternal separation activates microglial cells and induces an inflammatory response in the hippocampus of male rat pups, independently of hypothalamic and peripheral cytokine levels. Brain Behav Immun 55**:** 39-48.

223. Roque S, Mesquita AR, Palha JA, Sousa N, & Correia-Neves M (2014). The behavioral and immunological impact of maternal separation: a matter of timing. Front Behav Neurosci 8**:** 192.

224. Rouschop KM, Roelofs JJ, Sylva M, Rowshani AT, Ten Berge IJ, Weening JJ*, et al.* (2006). Renal expression of CD44 correlates with acute renal allograft rejection. Kidney Int 70**:** 1127-1134.

225. Roy-Chaudhury P, Khong TF, Williams JH, Haites NE, Wu B, Simpson JG*, et al.* (1996). CD44 in glomerulonephritis: expression in human renal biopsies, the Thy 1.1 model, and by cultured mesangial cells. Kidney Int 50**:** 272-281.

226. Rudemiller NP, Patel MB, Zhang JD, Jeffs AD, Karlovich NS, Griffiths R*, et al.* (2016). C-C Motif Chemokine 5 Attenuates Angiotensin II-Dependent Kidney Injury by Limiting Renal Macrophage Infiltration. Am J Pathol 186**:** 2846-2856.

227. Saleh MA, Boesen EL, Pollock JS, Savin VJ, Pollock DM (2010). Endothelin-1 increases glomerular permeability and inflammation independent of blood pressure in the rat. Hypertension 56**:** 942-949.

228. Saleh MA, Pollock JS, Pollock DM (2011). Distinct actions of endothelin A-selective versus combined endothelin A/B receptor antagonists in early diabetic kidney disease. J Pharmacol Exp Ther 338**:** 263-270.

229. Sallusto F, Geginat J, & Lanzavecchia A (2004). Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol 22**:** 745-763.

230. Sandberg K, & Ji H (2012). Sex differences in primary hypertension. Biol Sex Differ 3**:** 1-21.

231. Sanders BJ, & Anticevic A (2007). Maternal separation enhances neuronal activation and cardiovascular responses to acute stress in borderline hypertensive rats. Behav Brain Res 183**:** 25-30.

232. Satou R, Miyata K, Gonzalez-Villalobos RA, Ingelfinger JR, Navar LG, & Kobori H (2012). Interferon-γ biphasically regulates angiotensinogen expression via a JAK-STAT pathway and suppressor of cytokine signaling 1 (SOCS1) in renal proximal tubular cells. FASEB J 26**:** 1821-1830.

233. Scanzano A, & Cosentino M (2015). Adrenergic Regulation of Innate Immunity: A Review. Front Pharmacol 6**:** 1-18.

234. Schneider MP, Wach PF, Durley MK, Pollock JS, & Pollock DM (2010). Sex differences in acute ANG II-mediated hemodynamic responses in mice. Am J Physiol Regul Integr Comp Physiol 299**:** R899-906.

235. Schrader LI, Kinzenbaw DA, Johnson AW, Faraci FM, & Didion SP (2007). IL-6 Deficiency Protects Against Angiotensin II–Induced Endothelial Dysfunction and Hypertrophy. Arterioscler Thromb Vasc Biol 27**:** 2576-2581.

236. Schutte R, Huisman HW, Schutte AE, & Malan NT (2005). Leptin is independently associated with systolic blood pressure, pulse pressure and arterial compliance in hypertensive African women with increased adiposity: the POWIRS study. J Hum Hypertens 19**:** 535-541.

237. Schwaiger M, Grinberg M, Moser D, Zang JCS, Heinrichs M, Hengstler JG*, et al.* (2016). Altered Stress-Induced Regulation of Genes in Monocytes in Adults with a History of Childhood Adversity. Neuropsychopharmacology 41**:** 2530-2540.

238. Scott KM, Korff MV, Angermeyer MC, Benjet C, Bruffaerts R, De Girolamo G*, et al.* (2011). The association of childhood adversities and early onset mental disorders with adult onset chronic physical conditions. Arch Gen Psychiatry 68**:** 838-844.

239. Sen CK, & Packer L (1996). Antioxidant and redox regulation of gene transcription. FASEB J 10**:** 709-720.

240. Sharma JN, Al-Omran A, & Parvathy SS (2007). Role of nitric oxide in inflammatory diseases. Inflammaopharmacology 15**:** 252-259.

241. Shesely EG, Maeda N, Kim HS, Desai KM, Krege JH, Laubach VE*, et al.* (1996). Elevated blood pressures in mice lacking endothelial nitric oxide synthase. Proceedings of the National Academy of Sciences 93**:** 13176-13181.

242. Shrewsbury V, & Wardle J (2008). Socioeconomic Status and Adiposity in Childhood: A Systematic Review
of Cross-sectional Studies 1990–2005. Obesity 16**:** 275-284.

243. Silverman MN, & Sternberg EM (2012). Glucocorticoid regulation of inflammation and its functional correlates: from HPA axis to glucocorticoid receptor dysfunction. Ann N Y Acad Sci 1261**:** 55-63.

244. Souza AC, Tsuji T, Baranova IN, Bocharov AV, Wilkins KJ, Street JM*, et al.* (2015). TLR4 mutant mice are protected from renal fibrosis and chronic kidney disease progression. Physiol Rep 3.

245. Spradley FT, De Miguel C, Hobbs J, Pollock DM, & Pollock JS (2013). Mycophenolate mofetil prevents high-fat diet-induced hypertension and renal glomerular injury in Dahl SS rats. Physiol Rep 1**:** e00137.

246. Sriramula S, Haque M, Majid DSA, & Francis J (2008). Involvement of Tumor Necrosis Factor-α in Angiotensin II–Mediated Effects on Salt Appetite, Hypertension, and Cardiac Hypertrophy. Hypertension 51**:** 1345-1351.

247. Sriskandan S, Evans TJ, & Cohen J (1996). Bacterial superantigen-induced human lymphocyte responses are nitric oxide dependent and mediated by IL-12 and IFN-gamma. J Immunol 156.

248. Sturgis LC, Cannon JG, Schreihofer DA, & Brands MW (2009). The role of aldosterone in mediating the dependence of angiotensin hypertension on IL-6. Am J Physiol Regul Integr Comp Physiol 297**:** R1742-R1748.

249. Su S, Wang X, Kapuku GK, Treiber FA, Pollock DM, Harshfield GA*, et al.* (2014). Adverse childhood experiences are associated with detrimental hemodynamics and elevated circulating endothelin-1 in adolescents and young adults. Hypertension 64**:** 201-207.

250. Su S, Wang X, Pollock JS, Treiber FA, Xu X, Snieder H*, et al.* (2015). Adverse childhood experiences and blood pressure trajectories from childhood to young adulthood: the Georgia stress and Heart study. Circulation 131**:** 1674-1681.

251. Suglia SF, Clark CJ, Boynton-Jarrett R, Kressin NR, & Koenen KC (2014). Child maltreatment and hypertension in young adulthood. BCM Public health 14.

252. Taddei S, Virdis A, Ghiadoni L, Magagna A, & Salvetti A (1998). Vitamin C improves endothelium-dependent vasodilation by restoring nitric oxide activity in essential hypertension. Circulation 97**:** 2222-2229.

253. Taherzadeh Z, VanBavel E, de Vos J, Matlung HL, van Montfrans G, Brewster LM*, et al.* (2010). Strain-dependent susceptibility for hypertension in mice resides in the natural killer gene complex. Am J Physiol Herat Circ Physiol 298**:** H1273-H1282.

254. Tang D, Kang R, Coyne CB, Zeh HJ, & Lotze MT (2012). PAMPs and DAMPs: Signal 0s that Spur Autophagy and Immunity. Immunol Rev 249**:** 158-175.

255. Tata DA (2012). Maternal separation as a model of early stress: effects on aspects of emotional behavior and neuroendocrine function. Hellenic Journal of Psychology 9**:** 84-101.

256. Taylor LE, Gillis EE, Musall JB, Baban B, & Sullivan JC (2018). High-fat diet-induced hypertension is associated with a proinflammatory T cell profile in male and female Dahl salt-sensitive rats. Am J Physiol Heart Circ Physiol 315**:** H1713-H1723.

257. Therrien FJ, Agharazil M, Lebel M, & Lariviere R (2012). Neutralization of tumor necrosis factor-alpha reduces renal fibrosis and hypertension in rats with renal failure. Am J Nephrol 32**:** 151-161.

258. Thurston RC, Chang Y, Derby CA, Bromberger JT, Harlow SD, Janssen I*, et al.* (2014). Abuse and Subclinical Cardiovascular Disease among Midlife Women: The Study of Women's Health Across the Nation. Stroke 45**:** 2246-2251.

259. Tinsley JH, South S, Chiasson VL, & Mitchell B, M. (2010). Interleukin-10 reduces inflammation, endothelial dysfunction, and blood pressure in hypertensive pregnant rats. Am J Physiol Regul Integr Comp Physiol 298**:** R713-R719.

260. Tottenham N, Hare TA, Millner A, Gilhooly T, Zevin J, & Casey BJ (2011). Elevated Amygdala Response to Faces Following Early Deprivation. Dev Sci 14**:** 190-204.

261. Tran LT, MacLeod KM, & MbNeill JH (2009). Chronic etanercept treatment prevents the development of hypertension in fructose-fed rats. Mol Cell Biochem 330**:** 219-228.

262. Trott DW, Thabet SR, Kirabo A, Saleh MA, Itani A, Norlander AE*, et al.* (2014). Oligoclonal CD8+ T cells play a critical role in the development of hypertension. Hypertension 64**:** 1108-1015.

263. Tsuda K, Kimura K, & Nishio I (2002). Leptin improves membrane fluidity of erythrocytes in humans via a nitric oxide-dependent mechanism—an electron paramagnetic resonance investigation. Biochem Biophys Res Commun 297**:** 672-681.

264. Tuttle RS, & Boppana DP (1990). Antihypertensive effect of interleukin-2. Hypertension 15**:** 89-94.

265. van den Broek T, Borghans JAM, & van Wijk F (2018). The full spectrum of human naive T cells. Nat Rev Immunol 18**:** 363-373.

266. van Heuven-Nolsen D, De Kimpe SJ, Muis T, van Ark I, Savelkoul H, Beems RB*, et al.* (1999). Opposite Role of Interferon-γ and Interleukin-4 on the Regulation of Blood Pressure in Mice. Biochem Biophys Res Commun 254**:** 816-820.

267. Vetulani J (2013). Early maternal separation: a rodent model of depression and a prevailing human condition. Pharmacol Rep 65**:** 1451-1461.

268. Virdis A, Neves MF, Amiri F, Touyz RM, & Schiffrin EL (2004). Role of NAD(P)H oxidase on vascular alterations in angiotensin II-infused mice. J Hypertension 22**:** 535-542.

269. Vuurmans TJL, Boer P, & Koomans HA (2003). Effects of Endothelin-1 and Endothelin-1 Receptor Blockade on Cardiac Output, Aortic Pressure, and Pulse Wave Velocity in Humans. Hypertension 41**:** 1253-1258.

270. Walker DQ, Francis R, Cabassa J, & Kuhn CM (2000). Effect of Ovarian Hormones and Estrous Cycle on Stimulation of the Hypothalamo-Pituitary-Adrenal Axis by Cocaine. The Journal of Pharmacology and Experimental Therapeutics 297**:** 291-298.

271. Wallace K, Cornelius DC, Scott J, Heath J, Moseley J, Chatman K*, et al.* (2014). $CD4^{+}$T cells are important mediators of oxidative stress that cause hypertension in response to placental ischemia. Hypertension 64**:** 1151-1158.

272. Wang Q, Dong X, Wang Y, Liu M, Sun A, Li N*, et al.* (2017). Adolescent escitalopram prevents the effects of maternal separation on depression- and anxiety-like behaviours and regulates the levels of inflammatory cytokines in adult male mice. Int J Dev Neurosci 62**:** 37-45.

273. Weber DS, Rocic P, Mellis AM, Laude K, Lyle AN, Harrison DG*, et al.* (2005). Angiotensin II-induced hypertrophy is potentiated in mice overexpressing p22phox in vascular smooth muscle. Am J Physiol Heart Circ Physiol 288**:** H37-H42.

274. Wieck A, Andersen SL, & Brenhouse HC (2013). Evidence for a neuroinflammatory mechanism in delayed effects of early life adversity in rats: relationship to cortical NMDA receptor expression. Brain Behav Immun 28**:** 218-226.

275. Wink DA, Hines HB, Cheng RY, Switzer CH, Flores-Santana W, Vitek MP*, et al.* (2011). Nitric oxide and redox mechanisms in the immune response. J Leukoc Biol 89**:** 873-891.

276. Wright HL, Moots RJ, Bucknall RC, & Edwards SW (2010). Neutrophil function in inflammation and inflammatory diseases. Rheumatology (Oxford) 49**:** 1618-1631.

277. Wu J, Thabet SR, Kirabo A, Trott DW, Saleh MA, Xiao L*, et al.* (2014). Inflammation and mechanical stretch promote aortic stiffening in hypertension through activation of p38 mitogen-activated protein kinase. Circ Res 114**:** 616 625.

278. Xue Q, Yan Y, Zhang R, & Xiong H (2018). Regulation of iNOS on Immune Cells and Its Role in Diseases. Int J Mol Sci 19**:** 3805.

279. Yang J, Zhang R, Lu G, Shen Y, Peng L, Zhu C*, et al.* (2013). T cell–derived inducible nitric oxide synthase switches off Th17 cell differentiation. J Exp Med 210**:** 1447-1462.

280. Yatim KM, Gosto M, Humar R, Williams AL, & Oberbarnscheidt MH (2016). Renal dendritic cells sample blood-borne antigen and guide T-cell migration to the kidney by means of intravascular processes. Kidney Int 90**:** 818-827.

281. Ygberg S, & Nilsson A (2012). The developing immune system - from foetus to toddler. Acta Paediatr 101**:** 120-127.

282. Young EA, Altemus M, Parkinson V, & Shastry S (2001). Effects of estrogen antagonists and agonists on the ACTH response to restraint stress in female rats. Neuropsycholopharmacology 25**:** 881-891.

283. Yu L, Moriguchi T, Kaneko H, Hayashi M, Hasegawa A, Nezu M*, et al.* (2017). Reducing Inflammatory Cytokine Production from Renal Collecting Duct Cells by Inhibiting GATA2 Ameliorates Acute Kidney Injury. Mol Cell Biol 37**:** 1-17.

284. Zafari AM, Ushio-Fukai M, Akers M, Yin Q, Shah A, Harrison DG*, et al.* (1998). Role of NADH/NADPH oxidase-derived H2O2 in angiotensin II-induced vascular hypertrophy. Hypertension 32**:** 488-495.

285. Zgraggen S, Ochsenbein AM, & Detmar M (2013). An important role of blood and lymphatic vessels in inflammation and allergy. J Allergy (Cairo) 2013.

286. Zhang J, Patel MB, Griffiths R, Mao A, Song YS, Karlovich NS*, et al.* (2014). Tumor necrosis factor-alpha produced in the kidney contributes to angiotensin II-dependent hypertension. Hypertension 64**:** 1275-1281.

287. Zhang J, Rudemiller NP, Patel MB, Karlovich NS, Wu M, McDonough AA*, et al.* (2016). Interleukin-1 Receptor Activation Potentiates Salt Reabsorption in Angiotensin II-Induced Hypertension via the NKCC2 Co-transporter in the Nephron. Cell Metab 23**:** 360-368.

288. Zhou JR, Xu Z, & Jiang CL (2008). Neuropeptide Y promotes TGF-beta1 production in RAW264.7 cells by activating PI3K pathway via Y1 receptor. Neurosci Bull 24**:** 155-159.

289. Ziegler E, Gueler F, Rong S, Mengel M, Witzke O, Kribben A*, et al.* (2006). CCL19-IgG Prevents Allograft Rejection by Impairment of Immune Cell Trafficking. J Am Soc Nephrol 17**:** 2521-2532.

290. Zung A, Bluemenfeld O, Shehadeh N, Gottfried OD, Rakover YT, Hershkovitz E*, et al.* (2012). Increase in the Incidence of Type 1 Diabetes in Israeli Children Following the Second Lebanon War. Pediatr Diabetes 14**:** 326-333.

APPENDIX A

IACUC Approval Form

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM
Institutional Animal Care and Use Committee (IACUC)

MEMORANDUM

DATE: 17-Apr-2015

TO: Pollock, Jennifer

FROM: 

Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

SUBJECT: **NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 17-Apr-2015.

Protocol PI: Pollock, Jennifer

Title: Stress Related Mechanisms of Hypertension Risk, Project 2: Early life stress induced pro-hypertensive mechanisms **Sponsor:** GEORGIA REGENTS UNIVERSITY (AUGUSTA)

Animal Project Number (APN): IACUC-09999

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Institutional Animal Care and Use Committee (IACUC)
CH19 Suite 403
933 19th Street South
(205) 934-7692
0019 FAX (205) 934-1188

Mailing Address:
CH19 Suite 403
1530 3rd Ave S
Birmingham, AL 35294-

APPENDIX B

Figures from other Studies

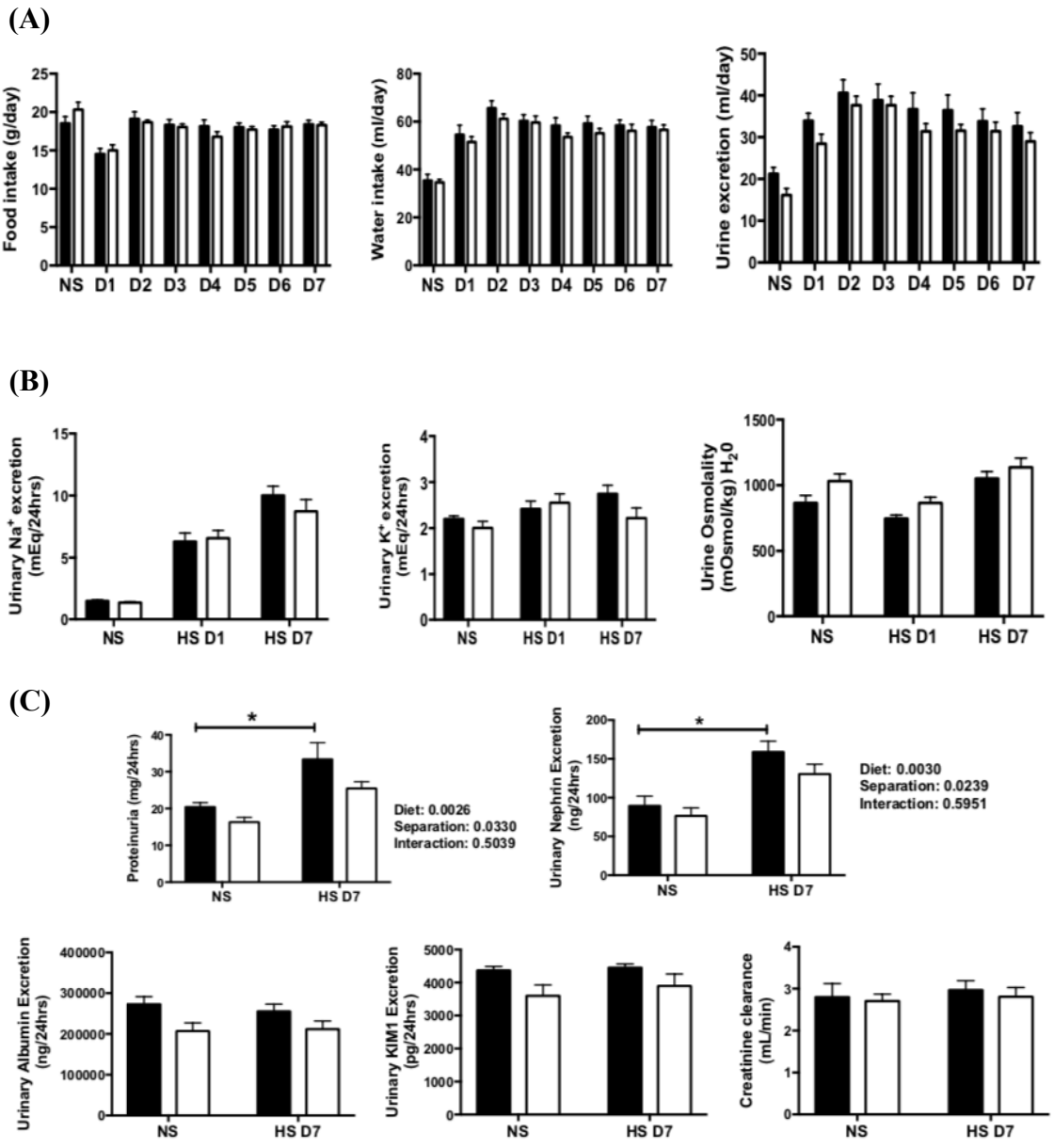


Figure 13: This study was conducted to determine if high salt diet (HSD) has an effect on sodium handling, glomerular and renal tubular damage in MatSep rats compared to control rats. (A) Food intake, water intake and urine excretion analyses. (B) Urinary sodium, potassium, and urine osmolarlity analyses. (C) Glomerular damage markers (nephrin and albumin), tubular damage markers (KIM1), and renal function as assessed by creatinine clearance and proteinuria.

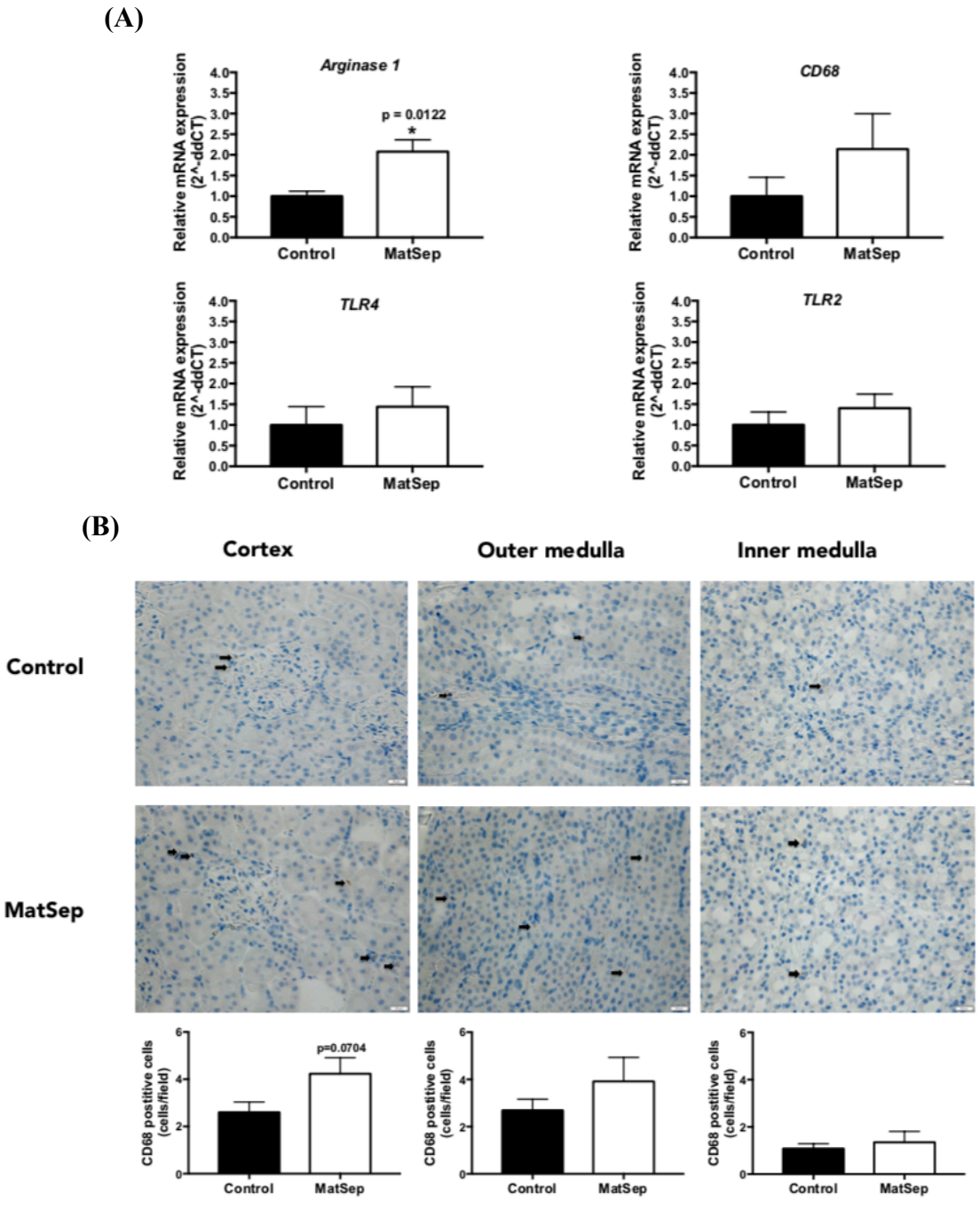


Figure 14: This graph shows the effect of high salt diet (HSD) on macrophage, macrophage polarization, and toll-like receptors (TLRs). (A) Gene expression of arginase 1 (a marker for M2 macrophage), CD68 (a marker for macrophages), TLR 4 and 2. (B) Immunohistochemical stain of CD68 positive cells in the cortex, outer medulla, and inner medulla of control and MatSep kidneys.

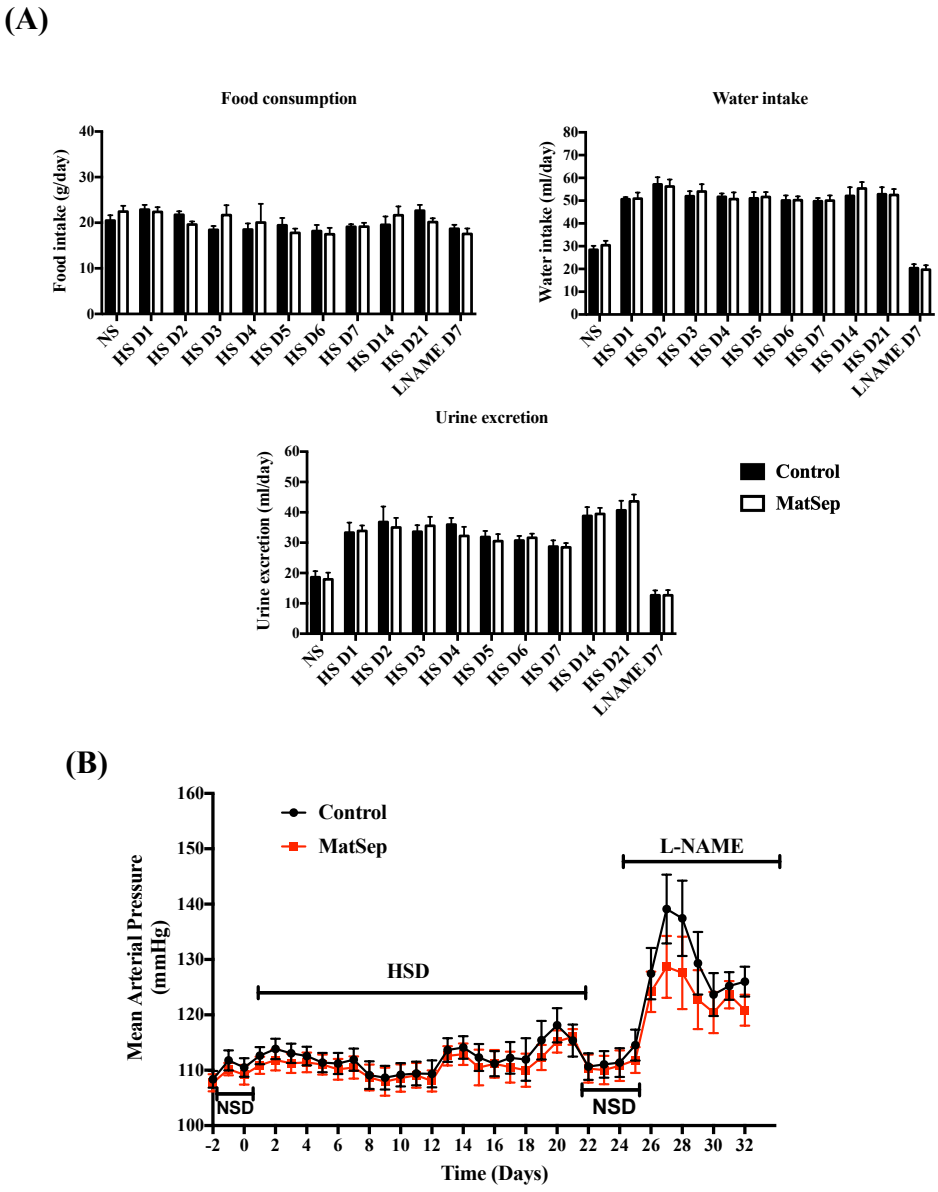


Figure 15: The aim of this study was to determine the effect of multiple insults on MatSep rats. Rats were first placed on a normal salt diet (NSD) for 3 days and then switched to a high salt diet (HSD) for 3 weeks. After which the animals were switched back to a NSD for 3 days and then given LNAME in drinking water for 7 days. (A) Food intake, water consumption, and urine excretion were assessed. (B) Telemetry blood pressure analyses for the duration of the treatment.